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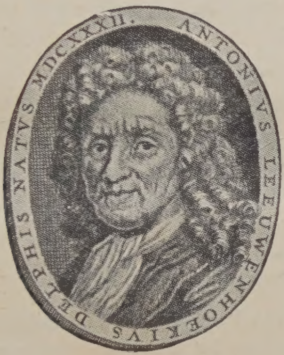
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# THE FORMATION AND CONSUMPTION OF LACTOBIONIC AND MALTOBIONIC ACIDS BY *PSEUDOMONAS* SPECIES

by

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(Received September 5, 1950).

## 1. INTRODUCTION.

In 1947 STODOLA and LOCKWOOD (1) published a study dealing with the production of bionic acids by various species of the genus *Pseudomonas* from lactose and maltose. They report that of fifteen species examined ten were able to produce acid from lactose. For almost all these strains, however, the acidification proved to be an extremely slow process, the only exception being *Pseudomonas graveolens*. This species was found capable of oxidizing lactose to calcium lactobionate, in rotating drums, with a yield of 75 per cent in 165 hours.

As for the oxidation of maltose it was again *Ps. graveolens* which proved to be the best of the eighteen species tested for the production of the corresponding maltobionic acid; a 77 per cent yield of calcium maltobionate was obtained in 50 hours in a rotating drum. *Ps. fragi* also oxidized maltose at a good rate, but the high calcium values obtained in the medium suggested oxidation beyond the bionic acid stage. The remaining species oxidized maltose too slowly to be of interest; the greater part of these species were, moreover, found to hydrolyze maltose.

The American authors rightly stress the importance of these observations, since they provide the first clear cut example of

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¹) J. D. L. gratefully acknowledges his indebtedness to the ,,Belgisch-Nederlands Cultureel Verdrag'' for a scholarship.

a biochemical oxidation of reducing disaccharides without prior hydrolysis.

There were several reasons which made it seem worth-while to extend these observations. In the first place, because the taxonomy within the genus *Pseudomonas* is still in a most unsettled state, so that it appeared quite possible that *Ps. graveolens* should be represented in other collections under a different specific name. Moreover, it seemed unlikely that *Ps. graveolens* would be unique amongst the numerous species of the genus in its ability of producing bionic acids in good yield. We, therefore, decided to test in this respect the *Pseudomonas* strains present in the collection of the Laboratory of Microbiology at Delft.

But also the theoretical implications of the observed "direct" oxidation of the two reducing disaccharides seemed to ask for further study. The remarkable fact, that here for the first time clear evidence had been obtained for a biochemical conversion of a disaccharide into a compound in which the oxygen bridge between the two hexose molecules had remained intact, seemed to suggest at first sight that the organism in question lacks the faculty of springing this bridge either by hydrolysis or phosphorolysis. Such an assumption, however, leads to the inacceptable conclusion that a bacterium growing at the expense of the disaccharide is able to convert this substrate into its normal cell constituents keeping the oxygen bridge intact. Already this consideration alone justifies the conclusion that also the bionic acid producing bacteria are able to bring about a hydrolysis — or phosphorolysis — of either the disaccharide itself or of some derivative formed from it. All the same, it seemed worth-while to investigate in how far bionic acids formed by some *Pseudomonas* species would be broken down in a later stage of the culture. This would mean that there is only a quantitative difference between species which are able to hydrolyze maltose and species like *Ps. graveolens*. It is evident that this question has also an indirect bearing on the much disputed problem of the so-called "direct fermentation" of disaccharides by yeasts. We refer herefor to the recent surveys by Hestrin (2, 3) and by Gottschalk (4).

## 2. PRELIMINARY OBSERVATIONS ON THE FORMATION OF ACID FROM GLUCOSE, MALTOSE AND LACTOSE BY *Pseudomonas* SPECIES.

First a selection of the various strains available in the collection was made. The strains were then streaked on plates containing yeast extract, 10% of the sugar to be tested, 1% finely divided calcium carbonate and 2% agar.

In pouring the plates care had been taken that the chalk was evenly suspended in the melted agar, and remained so on solidification of the agar sol. The plates were all examined after 48 hours incubation at 30° C.; first it was noted whether growth had occurred, and secondly whether acid formation as evidenced by more or less clear zones around the colonies had taken place. If not, this observation was repeated after another 48 hours incubation, but this did only rarely influence the outcome.

The results have been collected in Table I. It should be remarked that ten strains of doubtful identity which did not form acid from the disaccharides have been left out of the compilation.

TABLE I.

Growth and acid formation by *Pseudomonas* species on yeast agar, 10% sugar, 1% calcium carbonate.

| Species | Strain number | Growth on: | | | Acid formation on: | | |
|---|---|---|---|---|---|---|---|
| | | gluc. | malt | lact. | gluc. | malt. | lact. |
| *Ps. fluorescens* | 1—4 | + | + | + | + | — | ++ |
| „ *putida* | 2—1 | + | + | + | ++ | — | — |
| „ *putida* | 2—7 | — | + | + | — | — | — |
| „ *putida* | R | + | + | + | ++ | — | — |
| „ *punctata* | 3—1 | + | + | + | + | — | +? |
| „ *punctata* | 3—2 | — | + | + | — | + | — |
| „ *calco-acetica* | 4—1 | + | + | + | + | — | ++ |
| „ *tumefaciens* | 6—1 | + | + | + | — | — | — |
| „ *pyocyanea* | 7—4 | + | + | + | ++ | — | — |
| „ *devorans* | 8—1 | — | + | + | — | — | — |
| „ *aromatica* | 9—1 | + | + | + | + | ++ | + |
| „ *quercito-pyrogallica* | 9—2 | + | + | + | + | ++ | ++ |
| „ *acidovorans* | 12—1 | + | + | + | — | — | — |
| „ *zingiberis* | 13—1 | + | + | + | + | + | — |
| „ *chlororaphis* | 19—1 | + | + | + | + | — | — |
| „ *stutzeri* | 22—1 | + | + | + | — | — | — |
| „ *syncyanea* | 24—1 | + | + | + | + | — | — |
| „ *caryocyanea* | 28—1 | + | + | + | — | — | — |
| „ species | 17—1 | + | + | + | ++ | — | + |

It is perhaps good to remark that the lesser known species

*Ps. calco-acetica*, *Ps. aromatica* and *Ps. quercito-pyrogallica*[1]) have been described by BEIJERINCK (5) in his study on bacterial pigments as products of oxidation.

The results should be interpreted with considerable reserve. Even a superficial glance at the table suffices to conclude that by far the majority of the strains tested do not form any acid from the two disaccharides under the conditions of the experiment. This may mean that these sugars are not attacked at all, but it also seems possible that in these cases the oxidation of the sugar does not lead to the formation of intermediates of an acid character, only the final oxidation products: carbon dioxide and water being formed. Remarkably in a few cases no growth has occurred on the glucose containing plates, whilst there was distinct growth on the plates containing the disaccharides. It may, however, be dangerous to ascribe this to a preferential attack of the latter sugars; it can as well be that the higher molar concentration of the glucose used inhibited the growth by its osmotic effect.

However, from the point of view as outlined in the Introduction the following results are the most important. *Ps. quercito-pyrogallica* is characterized by a marked ability to form acid from both maltose and lactose, whilst with *Ps. aromatica* acid formation was more abundant on the maltose than on the lactose medium. *Ps. fluorescens* and *Ps. calco-acetica* produce acid from lactose, but not from maltose.

Nevertheless, it would be premature to conclude from these observations that in all these cases bionic acids have been formed. The acid formation may as well have been due to the formation of gluconic acid or of some other intermediate of an acidic nature formed after prior splitting of the disaccharide into its constituent hexoses.

### 3. THE FORMATION AND CONSUMPTION OF LACTOBIONIC ACID BY *Pseudomonas calco-acetica*.

In the preliminary plate test, reported in the previous section the acid formation from lactose by *Ps. calco-acetica* was especially marked. We decided therefore to start with a closer study of the lactose consumption by this species in a liquid medium.

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[1]) BEIJERINCK uses the name: *Pseudomonas aromatica* var. *quercito-pyrogallica*. We have thought it advisable to assign specific rank to this ,,variety'', and have accordingly changed its name.

Hereto the following medium was autoclaved in an Erlenmeyer flask of 500 ml: aqua dist. 15 ml, yeast extract 35 ml, calcium carbonate 1 g, $(NH_4)_2SO_4$ 0.2 g, $K_2HPO_4$ 0.1 g, $MgSO_4$ 7 aq. 0.025 g.

After sterilisation for 15 minutes at 115° C. 50 ml of an aqueous 20% lactose solution which had been sterilized by filtration through a Seitz filter was added. The flask was then inoculated with 5 ml of a 48 hours old culture of the organism in peptone water.

It seemed essential to provide plentiful aeration, and, therefore, in all experiments the culture flasks were placed in a shaking device in an incubator room at 30° C.

At certain intervals about 5 ml of the culture was taken out of the flask, and after centrifugation submitted to analysis.

In order to test whether at any moment monosaccharides were present in the medium use was made of BARFOED's reagent, as modified by TAUBER and KLEINER (6). Since it was found that the available lactose preparations contained small quantities of some BARFOED-reducing compound — presumably glucose — the aqueous lactose solution used in the preparation of the medium was freed from this substance by a heavy inoculation with a pure culture of *Saccharomyces cerevisiae*. After incubating the solution for three days at 30° C. the yeast cells were removed by centrifugation and the clear supernatant was sterilized by filtration through a Seitz filter.

The amount of lactose still present in the medium was determined by the reduction of FEHLING's liquor, using SCHOORL's iodometric method. The quantity of acid formed was established by determining the amount of calcium dissolved in the medium. Hereto we applied the method of KRAMER and TISDALL, using the prescription as given in WESTENBRINK's manual of physiological chemistry (7). Corrections were made for the initial calcium content of the medium.

Taking into account the bivalency of the calcium ion it is clear that each micromol of lactobionic acid formed will bring 0.5 microaequivalent calcium into solution. For this reason we have in all tables given the number of micro-aequivalents of calcium dissolved multiplied by 2, so that these figures can directly be compared with the micromols of sugar consumed. Equality of the figures in the two columns would, therefore, give a strong indication for a quantitative conversion of the sugar into the corresponding bionic acid. In Table II the results of a first experiment have been collected.

On considering the figures in Table II it is clear that on the whole

TABLE II.

Formation of lactobionic acid by *Pseudomonas calco-acetica* in a nutrient medium containing 10% lactose.

| Age of culture in days | Lactose present in micro-mols/ml | Lactose consumed in micro-mols/ml | Calcium dissolved in micro-aeq. × 2/ml | Lactobionate in % lactose consumed calcul. on Ca diss. |
|---|---|---|---|---|
| 0 | 264 | 0 | 0 | 0 |
| 1 | 178 | 86 | 70 | 81 |
| 2 | 84 | 180 | 160 | 89 |
| 3 | 21 | 243 | 206 | 85 |
| 4 [1] | 17 | 247 | 232 | 94 |
| 6 | 2 | 262 | 228 | 87 |

[1] After 4 days 2 grams calcium carbonate were added to the medium.

they fit in well with the assumption that the main conversion which the lactose has undergone is its oxidation to lactobionic acid. It is true that the calcium figures lag somewhat behind those for lactose consumption, but since certainly part of the lactose will have been consumed for assimilatory purposes this is not surprising. The conclusion that a rapid formation of lactobionic acid in a yield of 85—90% of the lactose has taken place finds support in the fact that in all stages monosaccharides were absent, as was evidenced by the negative outcome of the BARFOED-test.

On the other hand another possibility cannot be ruled out. It could also be that the lactose had primarily been split into glucose and galactose, and that these monoses did not accumulate in the medium, because they had at once been subjected to an oxidation, one of the components being oxidized completely to carbon dioxide and water, whilst the second had been converted into some acidic compound.

For this reason it seemed desirable to make certain that the acid formed in the culture was, indeed, lactobionic acid. Since a specific method for the determination of this acid is lacking, we had to have recourse to a more indirect method. Characteristic for the bionic acids is that as such they are unable to bring about a reduction of FEHLING's liquor. They can, however, be hydrolyzed by heating a solution duly acidified with mineral acid at 100° C.

After hydrolysis is complete one molecule of gluconic acid together with one molecule hexose have been formed from each molecule of the bionic acid. In this solution the hexose can then be determined after neutralisation with the aid of FEHLING's method.

We have, therefore, studied the conditions which determine the hydrolysis of lactobionic acid by diluted hydrochloric acid, and have found that a maximal reducing activity was attained by adding 1 ml 1.5 N HCl to 1 ml of the lactobionate containing solution, and heating the mixture in a test tube with reflux in a boiling water bath for one hour. Using culture media in a suitable stage a satisfactory agreement between the reducing activity and the amount of calcium dissolved was obtained.

In the experiment reproduced in Table III, in which 2 g calcium carbonate had been added to the medium, we have also applied this method for lactobionate determination.

It should be remarked, however, that this could only be done after the lactose had practically disappeared from the medium, because under the conditions described lactose too is hydrolyzed which also leads to an increase in reduction.

TABLE III.

Formation and consumption of lactobionic acid by *Pseudomonas calco-acetica* in a nutrient medium containing 10 % lactose.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| Age of culture in days | Lactose present in micromols/ml | Lactose consumed in micromols/ml | Calcium dissolved in microaeq. × 2 /ml | Lactobionate in micromols /ml | Lactobionate in % lactose consumed based on column 4 | Lactobionate in % lactose consumed based on column 5 |
| 0 | 271 | 0 | 0 | — | 0 | — |
| 2 | 63 | 208 | 194 | — | 93 | — |
| 3 | 24 | 247 | 242 | — | 98 | — |
| 5 | 8 | 263 | 250 | — | 95 | — |
| 8 | 7 | 264 | 252 | 248 | 95 | 94 |
| 16 | 6 | 265 | 276 | 218 | 104 | 82 |
| 23 | 4 | 267 | 280 | 204 | 105 | 76 |
| 32 | 1 | 270 | 282 | 194 | 104 | 71 |

On surveying the data collected in Table III the following can be concluded. In the first place it is striking that the lactose, notwithstanding its high initial concentration of approximately 10%, disappears so rapidly from the medium. Already after 3 days only a small amount remains. At that time there is a close agreement between the number of micromols lactose consumed, and twice the number of micro-aequivalents of calcium dissolved. As has already been remarked, this is a strong indication that the lactose was almost quantitatively converted into lactobionic acid. This agreement is also found in the determinations made after 5 and 8 days. Since after 8 days the lactose had practically completely been consumed, it became possible to apply also the bionate determination by hydrolysis. It is seen that this determination brought a most satisfactory confirmation of the assumption made, both methods for bionate determination yielding practically the same values.

The results obtained with the hydrolytic method in the later stages of the experiment leave, however, no doubt that the lactobionate is in the long run slowly disappearing from the medium. Nevertheless, in this phase the amount of calcium dissolved is still slowly increasing. To all probability this should be explained by accepting that a slow hydrolysis of lactobionic acid takes place, and that the hexose part which is set free is at once oxidized — since no increase in FEHLING reduction occurs — partly under formation of some compound of an acidic nature which leads to a further dissolution of calcium carbonate.

This conclusion is important, because it would imply that *Ps. calco-acetica* is invested with the ability to spring the oxygen bridge present in the lactobionic acid.

### 4. THE FORMATION AND CONSUMPTION OF LACTOBIONIC ACID BY *Pseudomonas quercito-pyrogallica.*

It seemed worth-while to extend the foregoing observations to another *Pseudomonas* species which in the preliminary plate test had proved to be endowed with a marked power of forming acid from lactose. As such *Ps. quercito-pyrogallica* seemed to be most suitable.

The experiments were made exactly in the same way as those described in the previous section. The results obtained in the first experiment, of a more preliminary character, are collected in Table IV.

Table IV again shows a rapid disappearance of the lactose and an almost corresponding increase in calcium dissolved into the

TABLE IV.

Formation of lactobionic acid by *Pseudomonas quercito-pyrogallica* in a nutrient medium containing 10 % lactose.

| Age of culture in days | Lactose present in micromols/ml | Lactose consumed in micromols/ml | Calcium dissolved in microaeq. × 2/ml | Lactobionate in % lactose consumed calcul. on Ca diss. |
|---|---|---|---|---|
| 0 | 249 | 0 | 0 | |
| 1 | 226 | 23 | 12 | 52 |
| 3 | 47 | 202 | 186 | 92 |
| 4 | 13 | 236 | 206 | 87 |

medium. Here too the results quite conform with the idea that the lactose is converted with a yield of about 90 % into lactobionic acid.

In order to get a confirmation of this view a second culture was started in which in the later stages lactobionic acid was also determined with the aid of the hydrolytic method. The results are given in Table V.

TABLE V.

Formation and consumption of lactobionic acid by *Pseudomonas quercito-pyrogallica* in a nutrient medium containing 10 % lactose.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| Age of culture in days | Lactose present in micromols/ml | Lactose consumed in micromols/ml | Calcium dissolved in microaeq. × 2 /ml | Lactobionate in micromols /ml | Lactobionate in % lactose consumed based on column 4 | Lactobionate in % lactose consumed based on column 5 |
| 0 | 275 | 0 | 0 | — | 0 | — |
| 2 | 69 | 206 | 196 | — | 95 | — |
| 3 | 20 | 255 | 254 | — | 100 | — |
| 5 | 10 | 265 | 266 | 246 | 100 | 93 |
| 8 | 9 | 266 | 256 | 238 | 96 | 90 |
| 16 | 23 | 252 | 278 | 232 | 110 | 92 |
| 23 | 17 | 258 | 310 | 250 | 120 | 97 |
| 32 | 7 | 268 | 338 | 282 | 126 | 105 |

The first stage of the experiment reported in Table V is very similar to the preceding experiments: the lactose is rapidly consumed and the amount of calcium dissolved is in good agreement with the assumption that the lactose is mainly converted into lactobionic acid. The hydrolytic determinations after 5 and 8 days brings a full confirmation that at least by far the greatest part of the acid formed is indeed a bionic acid. The data gathered after 16 days are, however, not so easily interpretable. In the first place there is an apparent increase in lactose which means that the ability to reduce FEHLING's liquor is increasing. This could at first sight find an easy explanation in a splitting of the earlier formed lactobionate into gluconate and the reducing hexose: galactose. Another part of this galactose might have been oxidized to some acid which could be responsible for the steady increase in dissolved calcium. Both observations seem to offer another proof that *Ps. quercito-pyrogallica* is able to split the oxygen bridge present in the lactobionate. It should, however, be acknowledged that these findings cannot be brought in agreement with the fact that the lactobionate figures based on the hydrolytic method are still increasing after 16 days. We are inclined to think that the two last figures in column 5 are due to faulty determinations, since it is difficult to accept that in this stage other metabolic products are formed which on hydrolysis yield FEHLING reducing compounds.

## 5. THE FORMATION AND CONSUMPTION OF MALTOBIONIC ACID BY *Pseudomonas quercito-pyrogallica.*

It was tempting to extend our experiences also to the formation of maltobionic acid, and with a view to the results reported in section 2 it seemed likely that *Ps. quercito-pyrogallica* would also prove to be a suitable organism in this respect.

The preliminary experiment was performed exactly in the same way as those reported in the previous sections, except that the 10% lactose in the medium was substituted by 10% maltose. Table VI gives a survey of the results obtained.

The results are most satisfactory from the point of view of maltose consumption: already after 3 days only 12% of the sugar is left, and after 6 days the sugar has practically disappeared from the medium. Moreover, the amount of calcium dissolved is at least of the same order as that of the maltose consumed. This means that in first approximation per micromol of maltose consumed one micro-aequivalent acid is formed which strongly suggests the ormation of maltobionic acid. However, the calcium figures are

TABLE VI.

Formation of maltobionic acid by *Pseudomonas quercito-pyrogallica* in a nutrient medium containing 10 % maltose.

| Age of culture in days | Maltose present in micro-mols/ml | Maltose consumed in micro-mols/ml | Calcium dissolved in micro-aeq. × 2/ml | Maltobionate in % maltose consumed calcul. on Ca diss. |
|---|---|---|---|---|
| 0 | 280 | 0 | 0 | 0 |
| 1 | 264 | 16 | 5 | 31 |
| 3 | 34 | 246 | 200 | 81 |
| 4 [1] | 18 | 262 | 206 | 79 |
| 6 | 9 | 271 | 248 | 93 |

[1] After 4 days 2 grams calcium carbonate were added to the medium.

not quite up to those of the maltose consumption, so that an albeit modest part of the maltose is evidently not converted into acid. It is difficult to explain the shortage in calcium dissolved solely on the basis of maltose assimilation, and it is, therefore, probable that *Ps. quercito-pyrogallica* is also endowed with a not negligible capacity to split maltose into the constituent glucose molecules and to oxidize these to non-acidic products.

Moreover, it is a remarkable fact that in the period between the fourth and the sixth day the amount of calcium dissolved still shows a rather considerable increase which cannot be accounted for by the negligible maltose consumption. The only possible explanation seems to be a splitting of the primarily formed maltobionate with oxidation of the glucose moiety into a compound of acidic nature which might well be a second molecule of gluconic acid.

This complex situation made it most desirable to obtain further confirmation for the primary formation of maltobionic acid. Here again it was indicated to prove the presence in the medium of a compound which on acid hydrolysis yields a FEHLING reducing sugar in casu: glucose. Hereto the conditions necessary for a practically complete hydrolysis of maltobionate were worked out. It was found that the same conditions as applied for the lactobionate hydrolysis could be maintained, with the exception, however, that the time of heating in the boiling water bath had to be extended to two hours.

The results obtained in the more definite experiment are collected in Table VII.

TABLE VII.

Formation and consumption of maltobionic acid by *Pseudomonas quercitopyrogallica* in a nutrient medium containing 10 % maltose.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| Age of culture in days | Maltose present in micromols/ml | Maltose consumed in micromols/ml | Calcium dissolved in microaeq. × 2 /ml | Maltobionate in micromols/ml | Maltobionate in % maltose consumed based on column 4 | Maltobionate in % maltose consumed based on column 5 |
| 0 | 271 | 0 | 0 | — | 0 | — |
| 2 | 177 | 94 | — | — | — | — |
| 3 | 141 | 130 | 114 | — | 87 | — |
| 5 | 101 | 170 | 136 | — | 80 | — |
| 8 | 22 | 249 | 182 | 170 | 73 | 68 |
| 16 | 9 | 262 | 174 | 178 | 66 | 68 |
| 23 | 4 | 267 | 192 | 176 | 72 | 66 |
| 32 | 1 | 270 | 226 | 142 | 84 | 53 |

It will be seen that the course of this experiment is for some reason or other somewhat different from that of the previous one, in so far as the maltose consumption proceeds markedly slower. But here too the amount of calcium dissolved lags behind the amount of maltose consumed, indicating that part of the maltose has been subject to another conversion than that of an oxidation to maltobionic acid. It is, however, quite satisfactory that in the analyses made after 8 and 16 days there is a good agreement between the maltobionic values calculated on the calcium figures and those obtained by acid hydrolysis. This seems to warrant the conclusion that in this stage about 68 % of the maltose consumed — and also of the maltose initially present — has, indeed, been converted into maltobionic acid. In the last two weeks, however, there is an unmistakable disappearance of the maltobionate, and since the calcium figures increase in the same period, it seems again very likely that the bionate is primarily split into gluconate and glucose, the latter being again oxidized, partly under formation of some acidic compound which may well again be gluconate.

### 6. GROWTH AND LACTOBIONATE CONSUMPTION IN MEDIA IN WHICH THIS COMPOUND IS THE MAIN SOURCE OF CARBON.

We have made an attempt to establish whether the lactobionate producing *Pseudomonas* species are able to grow and to consume lactobionate in media in which this compound is the sole source of carbon besides the organic compounds present in the diluted yeast extract. Hereto we have isolated some lactobionate from a large scale experiment with *Ps. quercito-pyrogallica*. The product was reasonably pure in so far that the results of the calcium determination and the hydrolytic bionate determination differed only 2.5%. It was found that on our usual agar medium in which the sugar had been substituted by 5% of the lactobionate growth of both *Ps. calco-acetica* and *Ps. quercito-pyrogallica* was much more abundant than that of control plates in the preparation of which the lactobionate had been left out.

In some experiments in liquid lactobionate media it was found that *Ps. quercito-pyrogallica* brought about a slow disappearence of lactobionate: about 20% was apparently consumed in ten days, as testified by hydrolytic bionate determinations. On the contrary· no lactobionate was consumed under these conditions by *Ps. calco-acetica*.

In concluding we will not omit to remark that we found several other micro-organisms which consume lactobionate very rapidly. This point will be dealt with in a forthcoming paper by the second author.

## Summary.

It has been shown that amongst 19 strains of 16 different *Pseudomonas* species four formed an appreciable amount of acid on a solid medium containing maltose, whilst six did the same on a solid medium containing lactose.

For *Ps. calco-acetica* it was proved that this species brings about a rapid conversion of lactose into lactobionic acid (yield ± 85—90%). The same was found to hold for *Ps. quercito-pyrogallica* (yield ± 90 %), which species, moreover, is also able to convert maltose into maltobionic acid (yields varying between 65 and 80%). An investigation of later phases of these cultures showed that the primarily formed bionates slowly disappear. The two species in question are, therefore, able to spring the oxygen bridge between the two

constituent parts of the bionic acids, and this makes it very likely that they are neither entirely devoid of the ability to split the disaccharides into the constituent hexoses.

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# CYTOCHEMICAL OBSERVATIONS ON NUCLEIC ACIDS AND NUCLEOPROTEINS IN NITROGEN DEFICIENT MICRO-ORGANISMS

by

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The present paper is an introductory study of the behaviour of nucleic acids and nucleoproteins of micro-organisms in nitrogen deficiency and an extension of our former work on nitrogen deficiency in *E. coli* (21, 22, 23, 59). In this investigation the localisation and the change in the nucleic acid and nucleoprotein content of aerobic sporeforming bacteria and yeasts were followed by cytochemical observations. We studied especially the GRAM reaction, the nuclear apparatus, the cytoplasmic ribonucleic acids and the volutin granules.

## EXPERIMENTAL PART.

### 1. MICRO-ORGANISMS.

| | Origin. |
|---|---|
| *Hansenula anomala* | Prof. KLUYVER, Lab. of Microbiology, Delft, Netherlands |
| *Torula utilis* | Prof. VIRTANEN, Biochemical Lab., Helsinki, Finland. |
| Yeast (not identified, mycelium forming) | Isolated from sputum from pulmonary disease by Dr J. VANDEPUTTE. |
| *Bac.cereus* var. *mycoides* | Prof. KLUYVER. |
| *Bac.subtilis* (here called strain 8) | Prof. CUVELIER's Lab. of Microbiology and Bromatology, State University, Ghent, Belgium. |
| *Bac.cereus* | Prof. KLUYVER. |
| *Bac.subtilis* (here called strain 9) | Prof. KLUYVER. |

We are indebted to all these for their kindness to supply us with these strains.

## 2. Culture media.

They consisted always of two parts 1) one ml of a solution containing the N source in varying concentrations and 2) 9 ml of a solution containing the carbon source, phosphate and salts. These two fractions were sterilized separately and mixed afterwards.

a) Not identified yeast. Solution 2: glucose 1 %; $K_2HPO_4$ 0.1 %; $MgSO_4$ 0.1 %; $CaCl_2$ 0.01 %; NaCl 0.01 %; $FeSO_4$ trace. Solution 1: The concentrations of $NH_4Cl$ were such that after mixing the culture medium contained 100, 10, 1, 0.1 and 0.01 mg per 10 ml.

b) *Hansenula anomala.* Solution 2: sucrose 3 %; $K_2HPO_4$ 0.1 %; $MgSO_4$ 0.05 %; KCl 0.05 %; $FeSO_4$ 0.001 %; pH 6.85. Solution 1: The concentrations of $NaNO_3$ were such that after mixing the culture medium contained 20, 2, 1, 0.2 and 0.02 mg per 10 ml.

c) *Torula utilis.* Solution 2: sucrose 5 %; $MgSO_4$ 0.1 %; $CaCl_2$ 0.05 %; $KH_2PO_4$ 0.4 %; pH 6. Solution 1: final concentrations of $(NH_4)_2SO_4$ 70, 7 and 0.07 mg per 10 ml.

d) *Bac. cereus* var. *mycoides* and *Bac. subtilis* (str. 8). Solution 2: glucose 0.5 %; $K_2HPO_4$ 0.1 %; NaCl 0.5 %; pH 7.3. Solution 1: final concentration of peptone 1, 0.5, 0.1 and 0.05 %.

e) *Bac. cereus* and *Bac. subtilis* (str. 9). Solution 2: glucose 0.5 %; NaCl 0.5 %; $K_2HPO_4$ 0.1 %; $MgSO_4$ 0.04 %; pH 7.3. Solution 1: final concentration of peptone 2, 0.5, 0.1 and 0.04 %.

## 3. Procedure.

The micro-organisms were first cultured in media with the highest N concentration and inoculated from young cultures in the media with varying N concentration. The amount of solution introduced with the wire loop was too small to influence the concentration of N in the media lowest in content. The organisms were cultured either in test tubes, in Erlenmeyers so on a larger surface, or in shake cultures, always at 30° C. No variation in the results was observed. The cultures were investigated until they were in the maximum stationary phase. Every 12 or 24 hours smears were prepared and the reactions performed. The photomicrographs were taken with a Leica apparatus. Usual illumination, condensor n.a. 1.4, objectif 100 × n.a. 1.25, ocul. 10 × . Negatives on Kodak Panatomic X and positives on Gevaert Ultra-Hard. We are indebted to Prof. Dr Gillis, Laboratory of Analytical Chemistry, State University of Ghent for his kind permission to use the camera with belongings and to Dr J. HOSTE of the same laboratory for his valuable advice in making the photographs.

## 4. STAINING METHODS.

Only the reactions suitable for cytochemical investigation have been used.

a) GRAM stain. The function of the Mg-ribonucleoproteins (Mg-RNPr) in the GRAM reaction is well known since the work of HENRY, STACEY and their collaborators (27, 28, 29) .We used HUCKER's modification (45) of the GRAM reaction. In order to reveal a weak GRAM-positive reaction in many cases the effect of the complete reaction was compared with the effect when counterstaining was omitted.

b) FEULGEN reaction. The reaction of the SCHIFF reagens with desoxy-ribosenucleic acids (DRNA) after acid hydrolysis is attributed to the formation of $\omega$-hydroxy-laevulinic aldehyde (46), although other substances might give this reaction ( 10, 14, 47, 48). For criticism of the latter authors see, however, (6). We used DELAPORTE's modification (19). Only in yeasts, however, this method was successful in our hands. Although in bacteria one to four FEULGEN-positive bodies were actually seen, analogous bodies and granules were seen also without hydrolysis.

c) LÖFFLER methylene blue stain before and after hydrolysis. It is known by the work of VENDRELY and LIPARDY (57) that by hydrolysis with N HCl at 60° C. for 10 minutes ribosenucleic acids (RNA) are nearly completely hydrolyzed, while DRNA are mainly untouched. ROBINOW (42, 43) and others observed structures in the cytoplasm which are revealed by staining with GIEMSA, LÖFFLER methylene blue etc. after hydrolysis.The observed bodies are thought to be the nuclear apparatus of bacteria. We stained with LÖFFLER methylene blue following LEWIS (34), for 3 — 4 min. The smear was first airdried, fixed in 96 % alcohol (the length of time of fixation had no influence, mostly 10—30 min. was used) and hydrolyzed. A control without hydrolysis always accompanied the experiment proper.

d) Volutin reaction. The composition of volutin is not exactly known. Some authors believe that there is a difference between volutin and metachromatic granules (8). BRANDT and CASPERSSON (9) demonstrated that the granules laying round the vacuole contain nucleic acids. On the other hand it has been proved that the metachromatic granules laying round the vacuole consist of poly-hexametaphosphates (61-65). It is on the other hand a fact that also nucleic acids can occasion metachromasia (37). Brandt (8) demonstrated that metachromatic substances and RNA-volutin lay closely together. We could observe the same facts in our experiments. To demonstrate the presence of volutin in yeasts we used the method of Knaysi (30) *viz.*, a flooding with 1 % of a saturated alcoholic solution of methylene blue in water for 2 min. and 1 % methylene blue, 1 % acetic acid for 3 min. Volutin stains strongly blue in a faintly blue cytoplasm. These granules had turned reddish when observed again some days afterwards.

## Results.

### 1. Nuclear apparatus.

In all our experiments with Löffler methylene blue the same results were obtained: the nuclear apparatus was always visible, even in the cells lowest in N content. The pictures agree completely with those published in literature. As has already been described by other authors, bacteria were observed with 1—4 nuclei. Yeasts mostly had one nucleus, exceptionally two. The yeast nucleus was studied merely for the sake of comparison with the bacterial one. The nuclear apparatus of bacteria was still visible in cultures of different ages. We were unable to explain this discrepancy with Robinow's statement (43); we agree, however, with Peshkoff (39) and Boivin (5) who noticed nuclei in old cultures.

The control experiments, without hydrolysis, with yeasts revealed dark blue, large irregular fragments in a dark blue cell. With decreasing N content of the medium and increasing age of the culture the cytoplasm became stained more faintly and the granules were much smaller. In yeasts they could be clearly seen to be situated in about a circle or an ellipse. Bacteria in cultures with normal N content showed always a uniformly stained cell, without visible internal structures. The intensity decreased likewise with decreasing N content and increasing age. In N deficient cells often structures analogous to the nuclear apparatus and in the same place were noticed. The spores (probably arthrospores) of the unidentified yeast showed 2—5 nuclei.

### 2. GRAM REACTION.

It is a general rule that the aspect of the cell changes with increasing N deficiency. The normal cells were stained uniformly darkly without any internal visible structure. In incipient N deficiency the GRAM stain, although still uniform, was much weaker. When deficiency was more marked, one or more strongly GRAM-positive bodies were seen in a weakly GRAM-positive (blueish purple) cytoplasm. This was especially obvious when no counterstain was used. When counterstain was used it practically masked the weak GRAM-positive stain of the cytoplasm which turned a reddish purple, while the same GRAM-positive bodies occurred. Of the latter two kinds were observed: a larger body B (Fig. 2 and 2a) in *Hansenula* and *Torula* mostly centrally situated, irregular and situated at random in bacteria, not observed in the mycelium forming yeast; a second kind of GRAM-positive structures: the A bodies occurring from a few to about 40 in all species studied (Fig. 2 and 2a). These could be noted especially clearly in the mycelial threads of the unidentified yeast. The pictures obtained of the cells in extreme N deficiency agreed remarkably with those published by HENRY and STACEY (28) plate 11, Fig. 1, 2, 5 and 8.

The spores of the unidentified yeast in N deficiency showed granules unlike their nuclei. Of all the species investigated, the mycelial threads lost most of their GRAM-positive substance.

The older the cultures, the more they resemble each other irrespective of the original N content of the medium. The conversion of the GRAM stain in old cultures is already of common knowledge (20).

### 3. VOLUTIN.

Volutin was investigated only in *Torula* and *Hansenula*. The resemblance with the controls of the methylene blue reaction was striking and making further description unnecessary. It must be stressed that in N deficiency the volutin granules are situated round a central reddish place, the whole situated on the same spot as the GRAM-positive body of the B type. Apparently the small granules of the A type do not show a volutin reaction.

## DISCUSSION.

### 1. GRAM REACTION.

The fact that the GRAM-positive substance decreases in N deficiency is reminiscent of former experiments (21, 22, 23, 59), which showed that dispensable enzymes, mostly adaptive as lactase and saccharase decreased or disappeared when N was deficient. On the other hand energy liberating enzymes (respiratory enzymes, catalase, etc. (22)) decrease less or not at all. Clearly this loss must not be attributed to a Mg deficiency (27), since Mg was added in most of the culture media. KNAYSI (31) demonstrated that spores of *B. mycoides* develop in glucose-phosphate-acetate medium into GRAM-negative vegetative cells.

It is significant that we never succeeded in obtaining completely GRAM-negative cells, not even with *Torula* and *Hansenula*, where the N content was so small that there was only a scanty growth. This indicates that the Mg-RNPr might have an important function. In this respect it must be noted that GALE *et al.* (26) observed a probable connection between GRAM reaction and amino-acid metabolism. "The GRAM-positive organisms are, in general, more exacting in their amino-acid requirements than GRAM-negative species" (51).

It remains an open question whether the protein part or the nucleic acid component of the complex suffered from N deficiency.

The microscopical pictures (see plate) strongly suggested the presence of a weakly GRAM-positive substance distributed evenly in the cytoplasm; BOIVIN (5) is of a same opinion. With the same reagents GRAM-negative cells such as coli and others, without coun-

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The figures illustrate some reactions in *Torula utilis*.

Fig. 1. GRAM stain. Ammonium sulphate 70 mg/10 ml. Uniformly stained cell. 2500 ×.

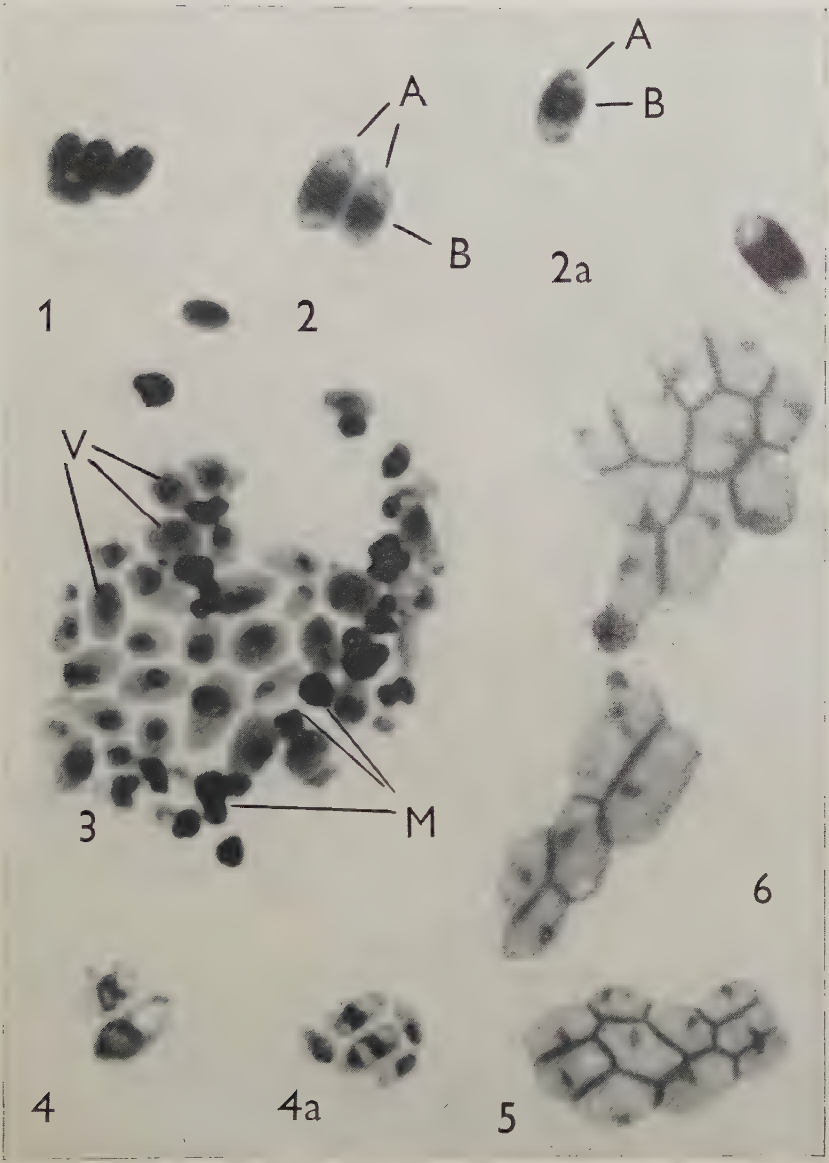
Fig. 2 and 2*a*. GRAM stain. Ammonium sulphate 0.07 mg/10 ml. 2900 ×. The cells show one greater central or excentric body B and many small granules A in a weakly GRAM-positive cytoplasm.

Fig. 3. Volutin reaction. Ammonium sulphate 70 mg/10 ml. Dark blue volutin granules in faintly blue cytoplasm. The granules M are precipitated methylene blue from the reagent. V = volutin. 2500 ×.

Fig. 4 and 4*a*. Volutin reaction. Ammonium sulphate 0.07 mg/10 ml. The cytoplasm is faintly blue. The volutin granules are situated in a circle round what is seemingly the vacuole. Note the resemblance in dimensions and situation with the body B from Fig. 2 and 2*a*. 2500 ×.

Fig. 5. Nucleus; Ammonium sulphate 70 mg/10 ml. Dark blue nucleus in faintly blue cytoplasm. 2900 ×.

Fig. 6. Idem but cells grown in 0.07 mg ammonium sulphate/10 ml. 3500 ×.


A
A
B
B
1
2
2a
V
M
3
6
4
4a
5

terstain, were always colourless or hardly perceptible, which proves that no artefact can be involved.

It has already been noted that in practically all cells deficient in N, small granules (A type) were observed. These granules are very similar, if not identical to those described by HENRY and STACEY (28) and others. It is striking that in the GRAM stain these granules always appear, whatever technique one uses: extraction with bile salts (28), acid alcohol (25), autolysis (25) and N deficiency. KNAYSI (32) demonstrated GRAM-positive granules in the GRAM-negative cytoplasm of *Thiobacillus thio-oxydans*; he attributed this to volutin. We are inclined to accept that the observed granules of the A type have another, may be only slightly different, composition of the GRAM-positive Mg-RNPr. In our opinion the possibility, although slight, remains, that they are staining artefacts. We endeavoured especially to show a possible relationship to volutin; 90—100 % of low-N cells showed the granules, while only 25—40 % showed one or two volutin granules beside the central volutin mass. It is thus highly improbable that these granules are volutin. They may be e.g. remnants of the GRAM-positive Mg-RNPr; or granules analogous to the mitochondria of the higher organisms or to the microsomes studied by CLAUDE (15), STERN (49), BRACHET *et al.* (6). In her review DELAPORTE (19) notes that most of the authors on bacterial cytology were unsuccessful in finding mitochondria, except ALEXEIEFF(1) and PETSCHENKO(40). The former author made the very interesting observation (p.416) that only bacteria which possess mitochondria take the GRAM. BRANDT (8) and BRANDT and CASPERSSON (9) demonstrated in yeasts with UV light granules, which thus contain nucleic acids, and which might function as centra where ribose nucleotides are formed. These granules consist of volutin. Whether for all of them this is the case has not been settled. LINDEGREN deems this view erroneous (35). LINDEGREN and LINDEGREN (36) took them for mixtures of lipoides with RNPr. In cells of *B. mycoides*, formed from spores in N free medium (33), granules were seen with the electron microscope. When unstained their size ranges from 0.03-0.17 micron to 0.08 – 0.1 micron, so at the visibility limit for the light microscope. It is possible that these too are analogous granules, which seem to increase in size, when surrounded with dye. Their electron scattering properties indicate that they also might contain nucleic acids. The authors state that they occur in the cytoplasmic membrane. Related granules were observed with the same species in the pre-sporal stage. Summarizing this discussion: it is not possible to establish for the present the true nature and function of these granules due to the lack of experimental evidence; they may be staining artefacts, remnants of the Gram-positive Mg-RNPr, or be related to mitochondria or microsomes. It is highly improbable that they consist of volutin. They certainly deserve further study.

In many experiments with bacteria it was clearly observed that Gram-positive structures were present at the place where with the technique of Robinow (see later) the nuclear apparatus is noted. It is unlikely that in the bacterial cell two different relatively large structures should be present on the same spot. It can thus be safely accepted that it is the bacterial nucleus itself which is stained Gram-positive. When one accepts that the Mg-RNPr are the only Gram-positive substances these must accordingly also be present in the bacterial nucleus. It is a well-known fact that nuclei of metazoan cells stain Gram-positive. It is known (6, 11) that in metazoan cells the nucleus forms ribose-nucleic acids at its wall and that the nucleus contains RNA in solution. The presence of ribosenucleic acids in or on the bacterial nucleus indicate that the nuclear apparatus has the same function as the one of higher organisms: it can form RNA which accumulates in the cytoplasm (perhaps also in the form of the observed Gram-positive granules) and functions as centre for the synthesis of proteins and enzymes (7, 12, 13). We must, however, bear in mind that *E. coli* may become Gram-positive by addition of highly polymerized DRNA (3) and that male gametes of *Ascaris megalocephala* contain a Gram-positive protein (38)[1].

The well-known phenomenon of the Gram conversion of dead cells has been explained by action of bacterial enzymes (52), ribonuclease (53, 4, 58). This may equally explain the conversion in old living cultures. Deussen (25) showed that by the formation of acid or alcaline fermentation products this conversion might occur. We made sure, by rigid control of pH, that this was not the case in our experiments. We offer, however, still another explanation.

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[1] Just after sending this paper to press an article of preliminary nature was published (P. Mitchell and J. Moyle, Nature **166**, 218 (1950)) in which the authors disagree with the opinion of Henry and Stacey (l.c.) and ascribe the occurrence of a positive Gram reaction to the presence of a phosphoric ester XP of unknown nature.

The older the cultures, the more numerous are the bacteria and the less N is available for new generations. The nitrogen content of the bacterial cell decreases actually with age (24). The bacteria assume more the low-N type in which less Mg-RNPr is formed. In performing the GRAM reaction a positive reaction is masked by using counterstain.

### 2. VOLUTIN.

It must be remembered that we have studied it only in yeasts. It is striking, that, like the GRAM strain, it did not disappear completely, even in cases with very slight growth. By BRANDT and CASPERSSON (9) and many others it has already been observed that volutin is located round and in the vacuole. The present observations agree with this view. In N deficiency the amount of volutin is greatly decreased, it is located then exclusively as a few metachromatic bodies round and in a central reddish place, most probably the vacuole. The observation that the GRAM stain (bodies of the B type) develops on the same spot where in N deficiency the vacuole and most of the volutin is present may be quite accidental, but it opens the question whether the volutin gives the GRAM reaction, as has already been pointed out by other authors (32).

### 3. NUCLEAR APPARATUS.

We always felt somewhat uncertain about the results, interpreted as giving support to the existence of the nucleus of bacteria. In fact it must be remembered that GIEMSA is a complex and unspecific mixture of dyes and that the use of enzymes (ribonuclease and desoxypolymerase) is as dangerous as it is useful: indeed we have only limited knowledge of their mode of action and many preparations contain impurities such as proteolytic enzymes (16, 44) and may be still others. The disaccord on the specificity of the FEULGEN reaction and its failure in many hands warned us against the interpretation of the results with the bacterial nuclei (compare e.g. the axial filaments in *B. mycoides* with FEULGEN (19) and the dumbbell shaped bodies seen with GIEMSA after hydrolysis (42)).

As has already been pointed out, we obtained the same pictures of the nuclear apparatus with methylene blue, irrespective of the N content of the medium. These observations give a support to the suggestion that these structures are the nuclei (41, 42, 43, 50, 53, 54, 55, 56 and others) since one might expect in N deficiency a reserve substance to disappear completely or at least decrease. Our results with volutin show, however, that the possibility of a reserve substance is not completely ruled out. In our opinion there still remains the possibility, however small, that these structures may be reserve substances, since our knowledge of nucleic acid metabolism in micro-organisms is slight. True, in many experiments described in literature, in the bacteria reserve substances were exhausted by preserving them some hours to one day in water, saline or buffer solution; but our experience (volutin control in the same conditions) showed that for many micro-organisms this length of time is insufficient to free the cells of reserve substances. Vigorous aeration may in some cases be a better method. It has already been shown by STILLE (50) that bacteria from agar with 0.1% peptone and 0.1% meat extract showed a normal picture with FEULGEN. It is stated that the observed structure could not be a reserve substance, but no control for the presence of such substance was reported. In our experiments the N content of the medium had decreased considerably under 0.04% and the experiments were continued until complete exhaustion of the medium.

### 4. CYTOPLASMIC NUCLEIC ACIDS.

The facts observed in the control experiments, that methylene blue is more readily taken up by normal cells and young cells, than by N deficient and old ones is partly known already: it suggests that cytoplasmic RNA decreases with age and nitrogen deficiency. During the preparation of the report of this work a paper was published by VIRTANEN and MIETTINEN (60) in which the total NA decrease along with N deficiency was proved by direct determination. We may therefore conclude that in their experiments it must have been the cytoplasmic RNA which decreases. These observations are equally in agreement with those of DAVIDSON and WAYMOUTH (18) and DAVIDSON (17) who noticed a decrease of RNA and a constancy of DRNA in starving liver, as well as with these of ANDREASEN (2) who noticed a fall in the NA phosphorus of the lymphoid organs of the rat in inanition.

## Summary.

1. Bacteria (*Bac. mycoides*, *Bac. subtilis*, *Bac. cereus*) and yeasts

(*Torula utilis, Hansenula anomala* and an unidentified mycelium forming yeast) were cultured in synthetic liquid media, with the same concentration of carbohydrate, phosphate and inorganic salts, but varying in amounts of nitrogen source. The concentration ranged between conditions allowing normal growth to extreme nitrogen deficiency (from 1/20 to 1/10.000 part of normal concentration). At regular intervals samples were taken and cytochemical reactions were performed. The cultures were studied until complete exhaustion of the medium.

2. When nitrogen is deficient the GRAM-positive character of all the investigated micro-organisms decreases. In stead of a uniformly darkly stained cell internal structures are revealed:
   1) a weak GRAM-positive cytoplasm.
   2) one or more larger GRAM-positive bodies which might be volutin.
   3) small granules, from a few to about 20—40, of unknown nature.
3. Even in extreme nitrogen deficiency the nuclear apparatus can still be noted, rendering it unprobable that the observed structures are reserve material.
4. The volutin decreases in nitrogen deficiency, small granules occurring round a central reddish vacuole. The same place stains GRAM-positive.
5. Cytoplasmic nucleic acids decrease in nitrogen deficiency.
6. The results are discussed.

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# THE ISOLATION OF THE THERAPEUTIC PRINCIPLE FROM A SURINAM NEGRO FOLK-MEDICINE

## ANTI-PARADENTOTIC EFFECT OF CIGAR-STUBS

by

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## INTRODUCTION

### 1. Aim of the work.

A few years ago, intending to investigate the bactericidal properties of vegetable juices and studying the literature devoted to this subject, I came upon data concerning tobacco and nicotine and their effects on bacteria and other living organisms. A person, who was later to be of great assistance to me, drew my attention to the fact that Professor Dr P. C. Flu had intended to start a similar research. Prof. Flu had noticed during his research work among the Maroons in Surinam that no paradentosis occurred there.

The Maroons usually have sound and strong white teeth and a healthy paradentium. Toothbrush and toothpaste are unknown to them; they clean their teeth and gums by rubbing them with cigar-stubs. Professor Flu thought there might possibly be some causal connection between the healthy condition of teeth and paradentium and the treatment with cigar-stubs. In his neighbourhood there happened to be some obstinate cases of paradentosis. At his request the patients massaged their teeth and gingivae with the ash end of a cigar-stub some three times a day. The effect was already noticeable within a few days; the swelling of the gums went down and secretion of pus discontinued; the dentist was amazed at the improvement. Unfortunately, the second world-war broke out and Professor Flu has not been able to give any further attention to this research. I therefore felt justified in taking up this investigation, which took almost four years.

Tobacco and tobacco smoke were formerly used as medicine. In Mexico the smoking of tobacco was considered a remedy for asthma, while tobacco leaves were used to cure ulcers and wounds. Tobacco taken as snuff was a remedy for headache. In the Braunschweiger Ratsapotheke of 1598 several recipes for tobacco preparations are given. Preparations from tobacco were herba, syrupus, oleum, unguentum and aqua nicotiniana (47). As late as in the last century an ointment of nicotine salicylate in a proportion of 0.1 to 2.5 % was used against scabies and several other parasitical diseases (56). On account of their high toxicity tobacco and nicotine are no longer used as medicine.

As the incidence of paradentosis is fairly high — according to HEINRICH (20) 14.2 % of the population suffers from this progressive rarefying process of the supporting tissues of the teeth — and its treatment presents many difficulties, at least when the preservation of the teeth is aimed at, there was a sound motive for starting this investigation, to find the therapeutic principle of the drugs concerned and if possible to formulate a medicine that might prove utilizable in practice.

## 2. THE CIGAR-STUB.

The cigar-stub contains concentrated substances originating from the combustion and dry distillation of tobacco. The nature and quantity of these products depends not only on the composition of the tobacco, but also on the rate of smoking and the moisture content of the tobacco (15).

BÖMER (7) records the following components of tobacco smoke: nicotine, ammonia, carbon dioxide, cyanides, thiocyanates, hydrogen sulphide, alcohols (such as methanol), aldehydes (such as methanal, ethanal, butanal), ketones (such as diethyl ketone, dipropyl ketone), acids (such as formic acid, acetic acid, butyric acid, benzoic acid, caproic acid), resins, etheral oils, myosmin, pyrrol, pyridine bases (such as pyridine, $\beta$-propyl-pyridine, picoline, collidine).

HENRY (21) mentions the following alkaloids of the genus *Nicotiana*: nicotine, nicotimine, anabasine, n-methylanabasine, isonicoteine, anatabine, 1-N-methylanatabine, nicotyrine, nicotelline, 2.3'-dipyridyl, nor-nicotine, nicotoine. The nicotoine has only been found in the Turkish type of tobacco. The occurrence of nicotyrine especially in the cigar type of tobacco has been recorded by WENUSCH (52).

The following alkaloids, apart from normal alkaloid constituents of tobacco, have been isolated by Wenusch and Schoeller (21) from tobacco smoke: Bases volatile in steam: myosmine, obeline, α-socratine, β-socratine, and γ-socratine. Bases non-volatile in steam: anodmine, lathreine and lohitam.

## 3. PARADENTOSIS.

The disease of paradentosis involves the rarefying of the supporting tissues of the teeth, especially of the alveolar bone (54, 31). Histologically the bone shows areas of osteolysis and a breakdown of the periodontal membrane (16, 40).

Leriche (28) drew up the laws which govern the physiology and pathology of the bone tissue:

1. The bone forms the mineral reserve of the organism. It is a physiological unit serving the internal environment. It supplies the organism immediately and at any moment with the mineral salts it needs. In physiological conditions decalcification of the bone sets in under the influence of the parathyroideal hormone. Recalcification follows immediately and is equal to the preceding loss of calcium. When decalcification exceeds the normal (for instance: through insufficient supply of calcium, a too high discharge of calcium, insufficient calcium-fixation for instance owing to hypovitaminosis, osteolysis through endocrine disturbances) it becomes visible and a pathologic state has set in.
2. The parathyroideal hormone and the other causes mentioned under 1. are not the only factors that cause decalcification. Hyperaemia, for instance, has the same effect but only locally. Optimum circulation, which probably varies individually, is essential to structural equilibrium of the bone. Most causes effecting hyperaemia decrease the calcium content.

Paradentosis has been subdivided in many different ways. Following the observations of Leriche, the simple division which has been adhered to by Vincent a.o. (48) is: *a.* inflammatory form, *b.* ischaemic form, *c.* a combination of forms *a* and *b*. We may assume that the bone resorption mentioned sub *a* is connected with the principle laid down in Leriche's second law. Vincent assumes that the cause of hyperaemia is a hematogenic infection localised in the paradentium as a result of an individual predisposition of the local terminal vascular system. He isolated the bacteria with the aid of a "hémoculture gingivale" (49). The resorption of the bone mentioned under *b* comes under Leriche's first law. A general decalcification is concerned, but according to Kölliker's law the bones which are being formed, and are the seat of physiologic development and exposed to heavy pressure, are the most strongly, and in a certain sense selectively, affected. The alveolar bone is in the latter conditions; hence resorption sets in before this is necessarily noticeable elsewhere. In many cases the combined form *c* is concerned, where the factors mentioned under *a* and *b* cooperate from the start or later on during the development of paradentosis. The hyperaemic and ischaemic forms occur to about an equal extent (48). Infections play an important part in 90 % of the cases of paradentosis (16). Indications of allergic reactions of the organism are also often found (23, 24).

Fosdick *et al.* (4, 5) pointed out that there is a connection between putrefaction in the oral cavity and paradentosis. They formulated a "putrefaction rate" as index of periodontal disease, which expresses the positive correlation between paradentosis and the degree of putrefaction. The products of putrefaction are due to the activity of all the oral bacteria together, the anaerobes in particular being mainly responsible (6).

## 4. Development of a hypothesis as a basis for experiment.

The present therapy of paradentosis is mainly based upon Kölliker's law. Antibiotics and chemotherapeutics have only a minor significance in the therapy of paradentosis. The activity of cigar-stubs must therefore be of a different and/or more complex nature. Considering what is known about paradentosis and about the chemical composition of the cigar-stub we drew up the following working hypothesis concerning the pharmacodynamic action of cigar-stubs in correlation with the pathological physiology of paradentotic disease:

1. The activity of the cigar-stub is of a local nature (a) and might probably be explained along the same line as Leriche's therapy of the pathological bone resorption (b).

2. This activity is enhanced by direct antibacterial effects on various pathogenic bacteria and on anaerobic oral bacteria.

This hypothesis is based upon the following considerations:

1a. We may assume that the effect of the tobacco product is not confined to the oral cavity. Strauss and Föckeler (45) found tobacco products in the dentine and in the cement of the teeth of smokers, even in the root canal and pulpa chamber. PINDBORG's statistical investigation (37) proves that the effect of these substances, which with normal smoking and tobacco chewing get into the oral cavity, is not wholly favourable to the teeth and paradentium. He found a positive correlation between the presence of tartar, gingivitis and the use of tobacco.

1b. LERICHE successfully treated osteolysis by inducing active hyperaemia by means of novocaine infiltration or through periarterial sympathectomy. Nicotine alkaloids primarily cause transient stimulation and secondarily a more persistent depression of all sympathetic and parasympathetic ganglia (17). This depression causing dilatation of the vessels is in agreement with the principle applied by LERICHE to his therapy of osteolysis. The principle of dilatation of the vessels as a therapeutic expedient is not limited to osteolysis, but has been applied for several affections. SCHMIDT (42), for instance, treated lupus erythematodes successfully with vasodilating agents. MOON (33) proved that vascular dysfunction is often the bottom of dermatosis.

The other pharmacological properties of nicotine: stimulation followed by depression of the central nervous system, of the skeletal muscles and of the blood pressure; the glandular effect, the effect on the heart and respiration, are not important for our hypothesis. In applying the cigar-stub as a therapeutic agent to the human being, these factors are, however, the first to be considered. The effects of tobacco-smoke and nicotine on the growth of young animals in utero and directly after birth (13), the infertility caused by tobacco-smoke must make us cautious in applying it to the human being (44).

2. It is a fact that both tobacco and tobacco-smoke have bactericidal properties. GOTSCHLICH (18) and MARCO (30) proved the bactericidal effects of tobacco and tobacco infusions. MILLER (32), TASSINARI (46), DUVAN (12), WOLFF (55) and PUNTONI (55) demonstrated the bacteriostatic and bactericidal effects of tobacco smoke *in vitro*.

MILLER used bacteria of decayed dentine; TASSINARI used *Vibrio cholerae*, *Klebsiella pneumoniae*, *Salmonella typhosa*, anthrax, *Staphylococcus aureus*, *Serratia marcescens* and *Mycobacterium tuberculosis*. DUVAN recorded the effect on pneumococci, *Corynebacterium diphtheriae*, *M. tuberculosis*, *Hemophilus influenzae* and staphylococci; he was not able to observe any effects on *Clostridium tetani*, streptococci, tetracocci and *Leptotrix buccalis*. WOLFF investigated the effects on *H. influenzae*, *C. diphtheriae*, pneumococci, staphylococci, *S. marcescens*, *V. cholerae*, *E. coli*, *S. typhosa* and *Shigella dysenteriae*. PUNTONI observed the effect of tobacco smoke on *Vibrio cholerae* meningococci, *H. influenzae, S. typhosa, C. diphtheriae,* streptococci and staphylococci. Huillain and Gy (39) found a highly bactericidal effect of tobacco smoke on streptococci and staphylococci, a weaker effect on *C. diphtheriae* and *Klebsiella pneumoniae.*

Körner (25) and Wernicke (53) added saliva to their experiments *in vitro* and report no influence on the effects of tobacco-smoke. Wernicke studied the effect on cholera vibrios, Körner the effect on bacteria from saliva, cholera vibrios, pyogenic streptococci and pneumococci. Puntoni passed smoke through water and proved that the water acquired bactericidal properties. When applying fractional distillation the first distillate containing formaldehyde appeared to be bactericidal against cholera vibrios, the second and third distillates inactive; the residue, however, giving a pyrrol reaction, again proved to be as bactericidal as the first distillate.

For the sake of completeness it should be mentioned that Arnold (3) proved the bacteriostatic action of hay smoke against streptococci, *S. typhosa, E. coli, C. diphtheriae* and staphylococci. The growth of *E. coli* was inhibited, while that of staphylococci was inhibited more strongly by the smoke of hay than by tobacco smoke. Serafani and Ungaro (43) proved the bactericidal activity of wood smoke.

Experiments *in vivo* in the oral cavity were made by Cavallara (11), Pinna (38), Appleton (2), Wolff (55) and Puntoni (55). Wolff investigated the effect on a culture of *Serratia marcescens* introduced into the mouth during smoking. The other investigators experimented on the normal mouth flora. They all observed that the number of bacteria decreased. Puntoni constructed a smoke chamber out of the intestine of a rabbit, and recorded the bactericidal effects of tobacco smoke on cholera vibrios and *S. typhosa.* Huillain and Gy (39) found that guinea pigs infected with *M. tuberculosis* succombed sooner to the effects of tobacco smoke than the control animals.

## LABORATORY EXPERIMENTS.

### 1. Products used for experimenting and their preparation.

The first experiments were carried out with the smoke of ordinary pipe tobacco burned in a clay pipe, and later ones with the smoke of burned tobacco waste. Combustion of these last products was effected in an electric oven made for this purpose with a capacity of 0.3 cubic metres. A mixture of oxygen and nitrogen in the proportion of 1 : 4 was passed through the oven at a rate of 3 litres per minute. The smoke was sucked off by means of a vacuum pump. Finally, experiments were made with Domingo tobacco. The leaves were burned in an ordinary small stove and the smoke sucked off by a vacuum pump.

In all the experiments the smoke was cooled in condensors from which the condensed matter was drawn off into flasks. The water was then removed by distillation and the remaining product was subjected to fractional distillation.

Domingo tobacco was used in order to obtain a product of more or less constant composition, which might serve as an object of comparison with the other products obtained from tobacco of unknown origin. Domingo tobacco was chosen for this purpose as this tobacco is more especially used for the manufacture of cigars, the starting point of this research being the cigar-stub.

Five fractions were split off. As the antibacterial effects of the various fractions were about the same, no further differentiation was effected. On account of secondary factors which will be discussed presently only fraction II was utilizable in the long run. Initially, an apparatus was used distilling at a pressure of 20 mm mercury and afterwards an improved apparatus in which a pressure as low as 0.2 mm could be obtained. The various fractions were thus, of course, distilled at a lower temperature, which had no disturbing influences on the experiments. The following fractions were isolated.

TABLE I.

Parts separated by fractionating tobacco smoke.

| Fraction | First series: Pressure 20 mm Hg | Second series: Pressure 0.2 mm Hg |
|---|---|---|
| I | till 110° | till 75° |
| II | 110°—160° | 75°— 90° |
| III | 160°—200° | 90°—105° |
| IV | 200°—250° | 105°—115° |
| V | 250°—300° | 115°—130° |

A solid tarry substance remained as residue in the flask.

In Table 2 a few data on some of the condensates are recorded. The nicotine percentage was estimated according to ROWAAN's method (41). The pH and the reduction potential were measured with a Beckman-meter. The last column will be discussed below.

TABLE II.

Properties of different parts of smoke condensate from two different kinds of tobacco.

| Kind of tobacco | Boiling point fraction (Pressure 0.2 mm) | pH | Reduction potential | Nicotine content | Boundary concentration to kill *E. coli* in 3 minutes |
|---|---|---|---|---|---|
| tobacco waste from cigarette tobacco without Domingo tobacco | 75°— 90° | 7.2 | –600 mV | 27.83 % | 4.5 % |
| | 90—105° | 7.4 | –610 mV | 20.56 % | 4 % |
| Domingo tobacco | 75°— 90° | 6.7 | –640 mV | 14.43 % | 2.5 % |
| | 90°—105° | 7.2 | –700 mV | 19.60 % | 3 % |

The condensate of Domingo tobacco smoke was divided into two portions.

TABLE III.

Various properties of the water-soluble and insoluble parts of Domingo tobacco smoke condensate.

| Domingo tobacco smoke | Boiling point fractions (Pressure 0.2 mm) | pH | Reduction potential | Nicotine content | Boundary concentration to kill *E. coli* in 3 minutes |
|---|---|---|---|---|---|
| Part of the condensate wich was dissolved in the water of condensation | 75°— 90° | 7.1 | –620 mV | 16.44 % | 2.5 % |
| | 90°—105° | 7.4 | –600 mV | 19.89 % | 4 % |
| Part of the condensate which was not dissolved in the water of condensation | 75°— 90° | 7.1 | –710 mV | 23.79 % | 1.5 % |
| | 90°—105° | 7.4 | –740 mV | 19.25 % | 1.5 % |

## 2. CHANGES IN THE PRODUCTS DURING STORAGE.

The composition and activity of fraction I were variable and it was therefore left out of consideration. In the other fractions we had to deal with:

A. Changes due to polymerization.

Fractions IV and V polymerized too strongly; they remained utilizable only for a short period. The movement in a horizontal capillary, the diameter of which was 0.25 mm, was studied. This width was chosen in view of the fact that the average width of the periodontal space in a normally functionating molar is 0.28 mm at its narrowest (31). The following results were obtained:

TABLE IV.

Decrease of the capillary activity of the smoke condensate.

| Boiling point of the fraction | Age of product kept at room temperature | Time required to cover the distance of 1 cm in a 0.25 mm capillary at room temperature |
|---|---|---|
| Fraction II | 24 hours | 37 seconds |
| 130° / 20 mm | 30 days | 40 seconds |
| Fraction II | 24 hours | 47 seconds |
| 170° / 20 mm | 30 days | 416 seconds |

B. Changes due to oxidation.

All the fractions were subject to oxidation and therefore discoloured in course of time. For this reason the products were kept at a temperature of —24° C., though no decrease in antibacterial activity at room temperature was observed. For clinical purposes an emulsion was made with de-activated gum arabic to which 1 : 1000 potassium metabisulphite had been added. Under these conditions the product proved stable for a long period: it retained its bactericidal properties, the viscosity of fraction II did not increase and the therapeutic effectivity remained stable for months.

### 3. INFLUENCE OF THE SMOKE CONDENSATE ON THE PRECIPITATION OF CALCIUM SALTS.

As paradentosis is a disturbance of the mutation of calcium in the periodontium, it was ascertained whether the smoke condensate might affect — in a simple way — the precipitation of calcium salts (26). If that were the case we should be concerned with a principle such as, for instance, has been applied by VINCENT in his therapy of paradentosis.

According to a method described by PFEIFFER (35, 36) glass dishes were made with a diameter of 9 cm by fixing with paraffin a glass ring on perfectly smooth window glass. The plates were placed exactly horizontal. A saturated solution of monocalcium phosphate to which calcium chloride had been added was poured into the dishes, so that the bottom was just covered with the solution. The dishes were left at room temperature and at the end of twelve hours the liquid had evaporated. A deposit of tricalcium phosphate was visible on the bottom.

Solutions were made of:

1. monocalcium phosphate
2. monocalcium phosphate with 0.25 % haemolized blood
3. monocalcium phosphate with 0.25 % haemolized blood and 0.25 % tobacco smoke condensate fraction II.

Plate I (photographs 1—3) show the results.

It is known that flower patterns originate as a result of a quick crystallization of the salt; the crystals having then no time to grow out normally in three directions. The following conclusions were drawn from an examination of the photographs.

1. The addition of haemolized blood to a solution of calcium phosphate accelerates the crystallization of the salt (photograph 1: calcium phosphate without blood; photograph 2: calcium phosphate with blood).
2. The addition of smoke condensate to a solution of calcium phosphate with haemolized blood gives rise to flower patterns besides some three-dimensional crystals (photograph 3: calcium phosphate with blood and condensate). So here the smoke condensate slightly delays the crystallization of the calcium salts.

Experiments in which saliva had been added to the solution produced analogous results. So the effects of the smoke condensate on paradentosis can not be accounted for by assuming that it has a stimulating effect on the crystallization of calcium salts.

## 4. THE EFFECTS OF TOBACCO SMOKE CONDENSATE AND OF NICOTINE ON THE PERIPHERAL BLOOD VESSELS.

A 4 % solution in alcohol of smoke condensate from Domingo tobacco (Fraction II) was used for the following experiments. The condensate contained 24 % nicotine. The solvent alone and a 1 % nicotine solution were used as a control. One drop of each of these

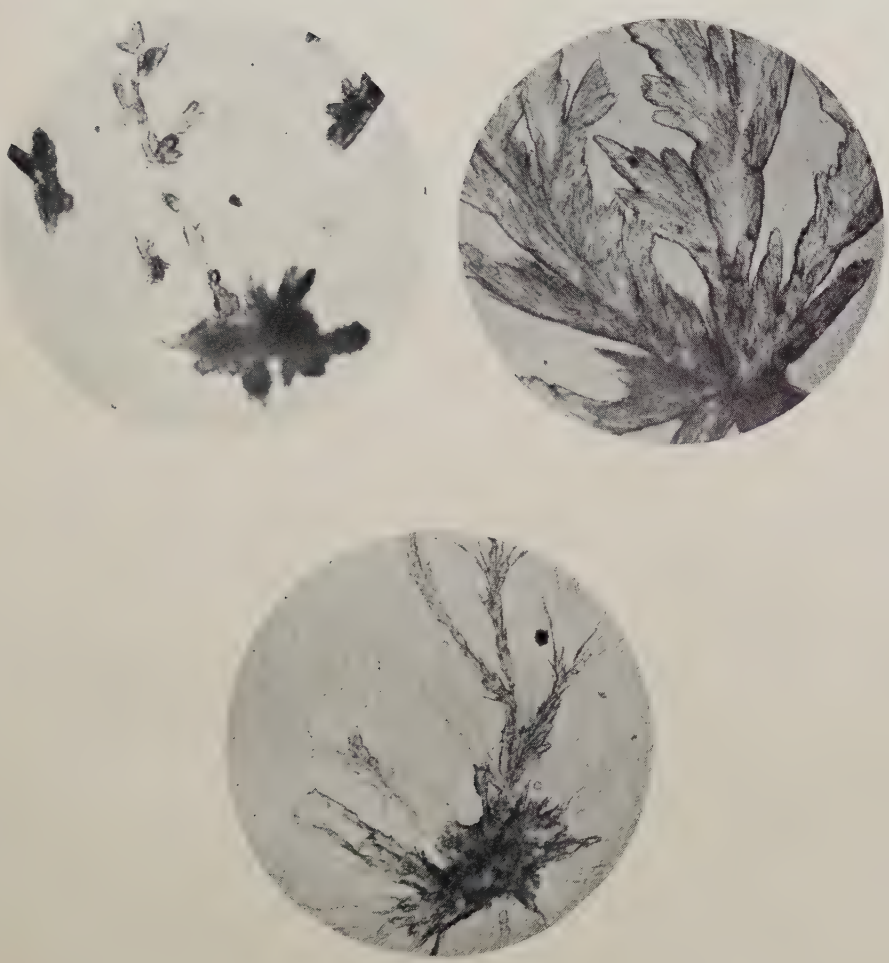

Plate I.

Deposit of tricalcium phosphate (magnification 100 ×).

Fig. 1. From a solution of monocalcium phosphate.

Fig. 2. From a solution of monocalcium phosphate with 0.25 % haemolized blood.

Fig. 3. From a solution of monocalcium phosphate with 0.25 % haemolized blood and 0.25 % tobacco smoke condensate.

liquids was applied to the web of a healthy frog. The effects were clearly evident after 5 minutes.

| | |
|---|---|
| solvent alone | no hyperaemia. |
| solvent with 1 % nicotine | strong hyperaemia. |
| solvent with 4 % smoke condensate | strong hyperaemia. |

### 5. EXPERIMENTS WITH TOBACCO SMOKE CONDENSATE AND WITH NICOTINE ON ANIMALS SUFFERING FROM PARADENTOSIS.

In order to ascertain whether the therapeutic activity of the cigar-stub perhaps depends exclusively on the presence of nicotine, three dogs suffering from paradentosis were taken under treatment; two with nicotine, one with smoke condensate fraction II. The products were emulsified in de-activated gum arabic. The gums of the dogs were smeared with the product twice a day. At the end of a month the treatment was discontinued. The results were the following:

TABLE V.

Results of treatment with nicotine and with smoke condensate on periodontal disease.

| Dog no. | Applied drug | Beginning of improvement after | Result after treatment for 1 month | Duration of the recovery after treatment was discontinued |
|---|---|---|---|---|
| 1 | nicotine 1 % | 14 days | no | — |
| 2 | nicotine 1 % | 12 days | less red gingivae | 7 days |
| 3 | smoke condensate fraction II 4 % | 5 days | gingivae normal | 4 weeks |

So nicotine has less effect, of shorter duration, on the affected gingivae than tobacco smoke condensate.

### 6. ANTI-BACTERIAL EFFECTS OF TOBACCO SMOKE.

#### A. Bacterostatic effects.

7 g of pipe tobacco were burned in a clay pipe for some 15 minutes. The smoke was sucked up by a water jet pump. The following experiments were carried out with the smoke.

1. The smoke was passed over agar plates 7.5 cm in diameter. Bacteria were inoculated on the plates. Streptococci, staphylo-

cocci, *V. cholerae* and *C. diphtheriae* served as test organisms. After the smoke had been passed over the plates the latter were incubated at 37° C. and examined after 24 hours. Result: not a single colony.

Comparative tests with other kinds of smoke:

a. 7 g of hay were burned in a similar way and passed over the agar plates. Results: no colonies.

b. The smoke of dried leaves of successively 7 g of *Xanthosoma maximiliana*, *Alocasia cuculata* and *Philodendron grandis* was passed as described above over the agar plates. Results: all organisms hàd given rise to colonies.

Experiments with superheated smoke: 7 g of tobacco were burned. Before being passed over the agar plates the smoke was conducted through a red-hot copper tube. Results: not a single colony.

2. The smoke was passed through a wash bottle containing 150 ml water. A few drops of this water were spread on an agar plate inoculated with staphylococci. The plate (diameter 9 cm) was incubated at 37° C. Results: The number of colonies amounted to about half that on the check plate.
3. The smoke was passed through two small wash bottles, one filled with alcohol and one filled with water, each of 75 g. Subsequently, the alcohol was entirely evaporated and the water was evaporated down to a third of the quantity. The aqueous solution smelled strongly of formaline. The residue after the evaporating of the alcohol was emulsified in 25 ml water. A few drops of each liquid were spread on inoculated agar plates. Results: Alcohol extract: growth of *V. cholerae*, staphylococci and *C. diphtheriae* was strongly inhibited. Aqueous extract: the same bacteria were less strongly inhibited in growth, although growth was slow.
4. The ash of 7 g of tobacco was shaken with water and then the entire wash was poured out on a plate inoculated with streptococci and with cholera vibrios. Next, the ash of 7 g of tobacco was shaken with alcohol, the alcohol evaporated and the residue dissolved in some ml of water and applied on similar plates. Results: in both cases the bacteria had grown normally.

B. Bactericidal effects.

The smoke of 14 g of tobacco was led through a 24-hour old broth culture of streptococci and through a broth culture of cholera vibrios. The duration of combustion was about 30 minutes. Then a few drops of the broth culture were inoculated on agar plates, and 24 hours later the result was observed. The following experiments were carried out:

1. The smoke was filtered through a wad of cotton wool before being passed through the broth. Results: the bacteria on the plates had grown normally.
2. The smoke was not filtered. Results: no colonies.
3. The ash of 14 g of tobacco was shaken with alcohol. The filtered alcohol was evaporated and the deposit suspended in 100 ml of a 24-hour old broth culture of *E. coli*. After 30 minutes a few drops of this culture were inoculated on agar plates. Results: the bacteria had grown normally.

### C. Summary.

Tobacco smoke has, in the above conditions, a bacteriostatic and bactericidal effect. Filtration of the smoke, i.e. the removal of the easily condensable products, destroys the bactericidal effect. The ash of tobacco has neither bacteriostatic nor bactericidal effects. Of the antibacterial product the following properties are known:

1. It is present in the smoke, but not in the ash of tobacco (Exp. A-1, B-2, A-4 and B-3).
2. It is proof against heating (Exp. A-1).
3. It is easily condensable (Exp. B-1 and B-2).
4. It dissolves more easily in alcohol than in water (Exp. A-3).

## 7. BACTERICIDAL EFFECTS OF CONDENSATION PRODUCTS OF TOBACCO SMOKE.

The smoke of 14 g of tobacco was condensed in a cooler. The condensate was collected and the water evaporated. A brown oily liquid remained.

30 drops of this oily smoke condensate, which had been obtained from 5 g of tobacco were added to 5 ml of a 24 hours' broth culture. After incubation at 37° C. some drops were spread on plates and the result read after 24 hours.

Staphylococci, streptococci, cholera vibrios, *C. diphtheriae, E. coli* and *Sh. dysenteriae* were used as test organisms. Results: all the bacteria were killed.

Next, the boundary concentration with a bactericidal effect in 24 hours was determined in a 24 hours' broth culture of bacteria.

Test bacteria were: *Sh. dysenteriae*, *S. typhosa*, *Proteus* and staphylococci. Results: The cultures of *Shigella*, *Salmonella* and *Proteus* remained sterile when they had been exposed for 24 hours to a smoke condensate in a concentration of 0.66 %. Against staphylococci the boundary concentration was 0.84 %.

### 8. DIFFERENCE IN BACTERICIDAL EFFECT BETWEEN THE SMOKE OF DIFFERENT KINDS OF TOBACCO.

These experiments were carried out with fractions II and III. The results are presented in table 2 (p. 36).

From the results we may conclude that the smoke from the burning of Domingo leaves has stronger anti-bacterial effects than the smoke of the burned tobacco waste, which, according to the statement of the manufacturer, contained no Domingo tobacco.

To ascertain whether these differences might depend upon the manner of burning, we made an experiment with 7 g of cigarette tobacco of the same composition as the used tobacco waste and with 7 g of cigar tobacco containing a large percentage of Domingo tobacco. Both samples were burned in clay pipes. The smoke condensate was collected and the concentration lethal to *E. coli* in three minutes was determined. For the cigarette tobacco the lowest bactericidal concentration appeared to be 5 %; for the cigar tobacco 3.5 %. Our experiment seems to indicate that the Domingo tobacco used for this purpose had a stronger bactericidal effect than the cigarette tobacco.

From table 2 it appears further that there was no direct connection between bactericidal effect and nicotine percentage nor between the bactericidal effect and the pH or the reduction potential. The experiments with the condensate from Domingo tobacco showed, moreover, that the part dissolved in the water of condensation was less bactericidal than the part not dissolved. The latter appeared to be soluble in alcohol. These results are in agreement with those of the bacteriostatic experiments with tobacco smoke as described on page 42 (Exp. A-3).

### 9. DIFFERENCE IN ACTIVITY BETWEEN THE FRACTIONS OBTAINED BY FRACTIONAL DISTILLATION.

Suspensions were made of fractions I, II, III, IV and V in a broth culture of bacteria by means of PETIT's solvent (70 ml glycerol, 90 ml distilled water and 40 ml alcohol 96 %). After 24 hours we ascertained the minimum concentration for effectivity. For all fractions the following percentages (table 6) were found to be effective.

TABLE VI.

Limit of bactericidal action of smoke condensate on bacteria in 24 hours.

| | | |
|---|---|---|
| *Escherichia coli* | | 0.66—0.8 % |
| *Staphylococcus aureus* | | 0.8 —1.26 % |
| *Thermobacterium acidophilus* | higher than | 1.26 % |
| Anaerobic oral bacteria | lower than | 0.66 % |

### 10. EFFECT OF THE SMOKE CONDENSATE ON ANAEROBES OF THE ORAL CAVITY.

The anaerobes were cultured according to the method indicated by HAMPP (19) for mouth treponemas. With a wire loop some material from the interstices between the teeth was transferred to a culture medium consisting of 0.2 % HUNTOON's agar with 0.1 % cysteine and 0.1 % fresh rabbit serum. By dark field illumination it was verified beforehand whether the material contained bacteria and mouth treponemas. One drop of the culture was pipetted into a flask containing the same medium, which was incubated anaerobically in a TADLOCK flask for 18 hours at 37° C. A few drops from this culture were inoculated into tubes containing the liquid medium to which smoke condensate was added in increasing concentrations. After anaerobic incubation at 37° C. for 18 hours a few drops from the tubes were inoculated in the well of a solid medium of the same composition, but with a higher percentage of agar.

This solid medium was found to contain GRAM-positive and GRAM-negative bacteria and many diplococci. When, however, we added more than 0.45 % smoke condensate to the liquid medium, only staphylococci grew in the inoculated well of the solid medium.

### 11. EFFECT OF THE SMOKE CONDENSATE ON PUTREFRACTION OF THE SALIVA.

This effect was studied by determining the sulphide percentage in the saliva by means of LORANT's method (29). In the saliva collected by paraffin stimulation according to the methods of BERG, BURRILL and FOSDICK (4, 5), the percentage of sulphides was determined. After an addition of 100 mg casein per 10 ml the saliva was shaken for three hours at 37° C. under anaerobic conditions and the percentage of sulphides again determined. Simultaneously, the same volume of saliva with casein to which 0.4 % smoke condensate had been added was incubated and shaken under the same conditions.

TABLE VII.

Effect of smoke condensate on the sulphide percentage in the saliva.

| Saliva sample No. | γ/ml sulphide in saliva | | |
|---|---|---|---|
| | without smoke condensate | | with 0.4 % smoke condensate |
| | initially | after 3 hours' incubation at 37°C. | after 3 hours' incubation at 37°C. |
| 1. healthy | 0.28 | 0.3 | 0.3 |
| 2. ,, | 0.5 | 0.5 | 0.5 —0.6 |
| 3. paradentosis | 0.6 | 1.2 | 0.5 —1 |
| 4. ,, | 0.4 | 0.8 | 0.4 |
| 5. ,, | 0.25 | 0.5 | 0.24—0.4 |
| 6. ,, | 0.3 | 2.0 | 0.33—0.5 |
| 7. ,, | 0.4 | 1.0 | 0.6 |

Therefore, the smoke condensate inhibits the effect of the enzymes inducing putrefactive changes in the saliva.

## UTILIZABILITY OF SMOKE CONDENSATE FOR CLINICAL EXPERIMENTS.

### 1. Index of toxicity.

To test the utilizability of smoke condensate as a possible antiseptic for the oral cavity we studied the inhibition of phagocytosis by means of slide-cells (14). A 24 hours' broth culture of staphylococci was diluted 1 : 676 and smoke condensate was added in various concentrations.

Defibrinated human blood was added in thrice the quantity of this diluted broth culture. The mixture was put into the slide-cells, which were incubated at 37° C. for 24 hours. Subsequently, the number of colonies of staphylococci in the cells with smoke condensate was compared with that in the cells without condensate.

At a condensate concentration of 0.4 % the number of colonies equalled that of the check. At a concentration of 0.5 % there were double that number. So 0.5 % of smoke condensate was taken to be the weakest dilution toxic to tissues.

We know that a dilution of 0.66 % of smoke condensate is lethal to anaerobes (table 6). The index of toxicity is therefore:

$$\frac{\text{highest dilution toxic to tissues}}{\text{highest dilution toxic to bacteria}} = 1.3.$$

This percentage compares favourably with most of the so-called antiseptic mouth-washes (50, 51), of which the $T_i$ practically always lies above 2.

## 2. Experiments on animals.

As the nicotine percentage of the condensate is about 20 % a fairly high toxicity was to be expected.

### *a.* Effect on guinea pigs.

A patch about 4 cm square of the skin on the back was shaved and rubbed with 1 ml of each of the condensates. Results were the following:

Fraction I: After 15 minutes the guinea pig was seized with spasms in the entire body, beginning in the hind legs; it fell on its side. After an hour the symptoms had passed off.

,, II: After 15 minutes violent spasms appeared, especially in the hind legs. These continued until the guinea pig died after 50 minutes.

,, III: After 15 minutes the guinea pig became restless, and then it lay down dazed. After one hour the symptoms had disappeared.

,, IV: After 15 minutes the guinea pig began to shiver slightly and did not eat anymore. No other symptoms were observed. After one hour its behaviour was normal again.

,, V: The guinea pig licked the patch that had been treated and seemed in no way affected.

### *b.* Effect of smaller doses on mice.

All the experiments were made with fraction II. A 20 g mouse was injected with 112 mg/kg. After 2 minutes the mouse died seized with clonic spasms. Next a mouse of 18 g was injected with 14 mg/kg. After 5 minutes it was seized with spasms and then fell into a stupor which lasted 50 minutes. In the next experiments no spasms were observed, only a stupor setting in during which the mouse sat as benumbed. The duration of this stupor is recorded in table 8.

TABLE VIII.

Action of smoke condensate injected in mice in moderate doses.

| Weight of the mouse | Quantity of smoke condensate fraction II injected (mg/kg) | duration of stupor in minutes |
|---|---|---|
| 18 g | 1.86 | 15 |
| 16.5 ,, | 4.27 | 30 |
| 17 ,, | 6.11 | 45 |
| 15.5 ,, | 8.73 | 90 |
| 17.5 ,, | 10.86 | 50 |
| 18 ,, | 25 * | 60 |
| 12 ,, | 20 * | 120 |

* with 1 % diparcol (22).

*c*. Experiments on mice with smoke condensate from which the nicotine had been eliminated.

To the smoke condensate fraction II picric acid was added until no further precipitation occurred. Four mice were injected with a dose of 20 mg/kg of this product. There was no phase of excitation and no spasms occurred. The mice did fall into a stupor which disappeared after an average of 40 minutes.

*d*. Experiments on a dog.

We injected a dog with a suspension of fraction II in a Petit mixture corresponding to 1 mg/kg smoke condensate. Convulsive movements followed at once, disappearing after three minutes. Then the dog lay in a stupor for 15 minutes. After about an hour the dog was normal again.

The same dog was treated with the same quantity of smoke condensate from which the nicotine had been removed. The dog did not respond to the injection.

*e*. Experiments on human beings.

Finally, the toxic effect was studied on human beings. Two ml smoke-condensate-emulsion of 4 % were spread on the gingivae of four persons. One person became dizzy and vomited; the other three showed no symptoms.

Conclusion.

The condensate has a highly toxic effect which is to be attributed to the nicotine. Diparcol inhibits this effect. The condensate also has toxic properties which are probably to be attributed to the alkaloids which are not soluble in water (10). Clinical experiments are only allowed if the necessary precautions are taken, while the patient is acquainted with the possible danger. Even when no immediate injurious symptoms appear the product must not be used a long time at a stretch.

### 3. THERAPEUTIC TRIALS ON HUMAN BEINGS.

In cooperation with a few dentists trials were made on a dozen patients suffering from paradentosis. As these patients lived all over the country and did not always present themselves for control, only a few of them could be regularly and accurately observed. Without any exception they all reported that they used the remedy with success. The redness of the gingivae subsided, exacerbations of the periodontal disease did not occur or were immediately checked and the hyperaesthesia of gingivae and teeth disappeared. When application of the remedy was discontinued the symptoms returned.

An account of a case in which a patient was treated with the smoke condensate is to be found on page 54.

The following preliminary conclusions were drawn from the clinical trials:

1. Tobacco smoke condensate is a therapeutic against periodontal disease. To ascertain in how far the various clinical forms are affected by the remedy further investigation is necessary.
   The following effects on patients were noted:
   a. the hyperaemic gingiva assumed a normal colour.
   b. exacerbations such as "real" pyorrhea and the formation of abcesses were checked.
   c. hyperaesthesia of gingivae and teeth disappeared.
   d. other oral infections were sometimes cured by the smoke condensate.
2. The smoke condensate — as applied in these cases — is not suitable for long and/or general use on account of its toxicity.

## THEORETICAL AND EXPERIMENTAL APPROACHES TO THE SYNTHESIS OF A NON-TOXIC REMEDY.

In view of our second conclusion — a conclusion drawn from our experiments on animals and on human patients — it was necessary to compose a therapeutic from which the toxic substances were eliminated as far as possible. It would have been possible to eliminate the components of the smoke condensate one by one, starting with the most injurious alkaloids and then to await the results of further experiments. This road would, however, have been a difficult and a long one; so another method was tried, based upon the principles stated in our hypothesis.

On account of the manner of preparation of the smoke condensate substances with a low boiling point such as e.g. formaline need not be considered. It was furthermore obvious that first of all attention must be paid to those substances which are peculiar to — in a quantitative sense — tobacco and tobacco smoke. Otherwise the Maroons might have hit on a less toxic product for the cleaning of their teeth and gums. Therefore the alkaloids in the tobacco and tobacco smoke were the first to be considered.

### 1. EFFECTS ON THE BLOOD VESSELS.

It is probable that the effect of smoke condensate on the blood vessels depends on the presence of nicotine. The effects of nicotine, however, are of a complex nature and it was obvious that more favourable results would be obtained with oxidation products of nicotine, such as nicotinic acid, which causes practically only dilatation of the blood vessels. To ascertain whether nicotinic acid lends itself to local application the following experiment on the ear of a rabbit was made:

Following the example of BOQUET, LEBAULD and GUICHARD (8) drops of a 30 % solution of nicotinic acid were applied to the shaved ear of a rabbit. Immediately the number of visible capillaries increased strongly, while the lumen of the arteries was dilated by about 18 %.

This experiment proved that the product penetrates the epithelium and exerts a dilating effect on the smaller blood vessels. It is noteworthy and may probably be important for the explanation of the therapeutical effect of the product, that after ten days the rabbit, which was in excellent condition, had a small hole in the ear on the spot where the nicotinic acid had been applied. Apparently the product in high concentrations has a lytic effect on the epithelium. We know that in paradentotic disease the epithelium of the gingival mucosa proliferates into the subepithelial tissue;

consequently the osteoblasts of the periodontal membrane near that spot can no longer form cement, for the bone tissue — for instance in cases of transplantation — only grows when at a distance from epithelium. The hole in the ear of the rabbit suggests the possibility that the drug, locally applied in a sufficiently high concentration, prevents the epithelium from proliferating, with the result that the apposition of cement remains possible.

### 2. ANTI-BACTERIAL EFFECTS.

The alkaloids of tobacco and tobacco smoke belong to the group of the pyridine alkaloids. ALBERT, RUBBO and BURVILL (1) traced a relationship between bacteriostatic action and chemical structure in this group. Two essential requirements for antibacterial activity were noted: adequate cationic ionization and an adequate area of flat surface.

Pyridine and its lower homologues do not have a strong bacteriostatic effect, the dimension of the pyridine nucleus as calculated from the tables of bond-distances by BRANCH and CALVIN being 3.97 × 4.38 Å and the molecule slightly ionized. Even when highly ionized, the molecules of the heterocyclic bases must possess a flat area above 28 sq.Å for powerful antibacterial properties to appear (1). The minimum flat area required is that which provides an area sufficiently large for adsorption on a biological surface. It has been shown that the atoms responsible for this adsorption should all lie in one plane. As to the nicotine molecule, it is known that the pyridine nucleus and the hydrated pyrrol nucleus do not necessarily lie in one plane. In spite of the strong basic properties of the nicotine molecule we may not expect a strong bacteriostatic effect. In fact our experiments proved that the water-soluble fractions of tobacco smoke are less bacteriostatic than the other fractions. The latter are weak bases and are present in the smoke condensate in much smaller quantities than nicotine. So, when looking in this group for an alkaloid which is more bacteriostatic than nicotine, we must choose one which possesses a larger flat area than pyridine or nicotine, therefore a pyridine alkaloid with a sufficiently large co-planar area built on to the molecule.

The facts known about the bacteriostatic drug are that:

1. it is found in tobacco-smoke, not in the ash;
2. it has a fairly high boiling point;
3. it is stable to high temperatures (250° C.);

4. it dissolves better in alcohol than in water;
5. the content of the drug is higher in the smoke of cigars than in the smoke of cigarettes, the cigars containing Domingo tobacco;
6. it is likely to have a large co-planar area built on the pyridine-nucleus.

With the aid of this knowledge we were able to trace its chemical structure.

All the properties required are possessed by the nicotyrine molecule. Further experiments proved this surmise to be correct. The co-planar area being a pyrrol nucleus will perhaps prove to be important. BORY (9) directed attention to the fact that the hetero-cyclic ring structures of five atoms might be of great use as therapeutics for human being. Pyrrol has long been known as a drug active against putrefaction (27).

An experiment was carried out with nicotine and nicrotyrine to study their relative effects on bacteria.

TABLE IX.

Effects of nicotine and nicotyrine on bacteria.

| Dilution | Examined substance | pH | Bactericidal in 30 minutes | Bacteriostatic |
|---|---|---|---|---|
| 1 : 50 | nicotine | 9 | — | + |
| | nicotyrine | 8.5 | + | + |
| 1 : 100 | nicotine | 8.9 | — | + |
| | nicotyrine | 8.4 | + | + |
| 1 : 200 | nicotine | 8.6 | — | + |
| | nicotyrine | 8.4 | — | + |
| 1 : 400 | nicotine | 8.4 | — | — |
| | nicotyrine | 8.4 | — | + |
| 1 : 800 | nicotine | 8.2 | — | — |
| | nicotyrine | 8.2 | — | — |

Nicotyrine is bactericidal in a concentration 1 : 100 and bacteriostatic in a concentration 1 : 400. Nicotine has no bactericidal properties, but is bacteriostatic in a dilution 1 : 200.

Another experiment was made with anaerobes of the oral cavity. Anaerobes of the oral cavity were cultured according to the method indicated by HAMPP. To this culture several products were added in various concentrations. Dilutions were made of the condensate of tobacco smoke fraction II, nicotine, nicotyrine, and of nicotyrine + nicotinic acid.

TABLE X.

Anti-bacterial effects of tobacco derivatives.

| Product | Boundary concentration for bacteriostatic action | Boundary concentration for bactericidal effect in 30 minutes |
|---|---|---|
| condensate of tobacco smoke (Domingo tobacco) | 1 : 110 | 1 : 110 not bactericidal |
| nicotine | 1 : 110 | 1 : 110 not bactericidal |
| nicotyrine | 1 : 220 | 1 : 110 |
| nicotyrine and nicotine acid | 1 : 440 | 1 : 110 |

### 3. Effects on putrefraction.

Finally, the effect of nicotyrine on putrefaction in the saliva was examined. An experiment was carried out with the saliva of four patients suffering from a high degree of putrefraction in the saliva.

TABLE XI.

Effect of nicotyrine on putrefaction in saliva.

| Saliva sample no. | γ/ml sulphide in saliva | | |
|---|---|---|---|
| | without nicotyrine | | 1% nicotyrine |
| | initially | after 3 hours incubation at 37° C. | after 3 hours incubation at 37° C. |
| 1 | 0.7 | 2.3 | 0.8 |
| 2 | 0.4 | 1.8 | 0.5 |
| 3 | 0.35 | 2.5 | 0.4 |
| 4 | 0.5 | 2.0 | 0.6 |

We may conclude that nicotyrine has the following pharmacological effects:

a. it is bacteriostatic, in higher concentrations bactericidal.

b. it inhibits the action of the enzymes giving rise to putrefactive changes in the saliva.

### 4. Toxicity of the synthetic remedy.

A solution was made containing nicotyrine and nicotinic acid, and to study the effect on higher organisms, mice were subcutaneously injected with a dose of the solution equal to 150 mg/kg nicotyrine. The mice injected after a few minutes fell into a sort of stupor. They lay quietly on their sides, but appeared to be able to walk. They gave the impression of being immediately tired by walking. They remained in this condition for some hours. After four hours the mice were again in a perfectly normal condition.

As a check, mice were injected with a product without nicotyrine. In this case the mice did not show any reaction. We may therefore assume that nicotyrine is adsorbed to the cells of the nervous system and has a more or less paralysing effect. These results may explain the fact that the hypersensitive teeth of patients suffering from periodontal disease grew less hyperaesthetic after the application of the solution.

The dose used in our experiments so far exceeds the therapeutic dose (about a hundred times), that we might without scruple experiment with the solution upon patients (34).

## CLINICAL TRIALS WITH PAREX 1).

### 1. Periodontal diseases.

A woman, aged 32 years, suffered for years from periodontal disease, which had set in after appendicitis. Six months previously a tooth has been lost through the disease. Tooth habits: grinding of the teeth during sleep.

Previous treatment: hydrogen peroxide, sulphathiazole.

Physical examination: Thyroid enlargement, nervousness, cold hands and feet; sensitive skin, varicose veins in the legs.

Dental and periodontal examination: second left mandibular molar lost.

Inflamed and easily bleeding gingivae, especially around the lower anterior teeth and the maxillary left molars.

Treatment with smoke condensate in a 4 % paste: After one month the gums were in a healthy condition, they were normally pink and firm. When the treatment was stopped the symptoms returned in a few weeks.

A few months later Parex was applied. The patient recovered from gingivitis in two days. The treatment was continued for 4 months. Then the patient discontinued the treatment and told the dentist that the periodontal disease had been cured. After the dentist had fixed an orthodontic appliance the gingivitis returned. Without removing the appliance and without another treatment the gingiva was treated with Parex three times a day. After four weeks the periodontium was in a normal condition again.

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1) For clinical use the name "Parex" was chosen for the product. All clinical trials were made with preparations manufactured by the laboratories of Keur & Sneltjes, Haarlem (Holland). I owe a special acknowledgement to them for their collaboration and especially for their patience which I so often sorely tried.

Other cases in which the patients were treated with Parex — also cases with X-ray examination — will be described elsewhere.

### 2. SUMMARY OF CLINICAL DATA.

The clinical experiments gave the following results:

1. the inflammation and hypertrophia of the gingiva disappeared by Parex treatment. The gingiva became hard, firm and pink.
2. hypersensitiveness disappeared.
3. exacerbations of a chronic periodontal disease were soon brought to a standstill and did not bring about extrusion of teeth.
4. chronic progressive resorption of the bone was inhibited.

### 3. CONCLUSION CONCERNING THERAPEUTIC USE.

Parex proved to be a useful remedy for the treatment of periodontal diseases. It cures the symptoms which irritate the patient: swelling, exudation, pain and tainted breath. As it inhibits bone resorption, it is very helpful in causal treatment of the periodontal disease. Combined with correctional treatment of local and systemic causes of the disease, it plays a part in bringing about restoration of normal function.

### 4. VARICOSE ULCERS AND OTHER DISEASES.

A woman, aged 56 years, suffered for two years from a varicose ulcer. A brownish red scaly skin encircled a rather deep tissue defect with purulent and malodorous secretion. Large convoluted veins were prominent when the patient stood upright. Six months previously the ulcer had been healed by rest and penicillin treatment. After some months the ulcer had returned. The patient refused penicillin treatment because of the pain involved, and her employment did not allow of her taking a rest-cure.

Treatment with Parex: Parex was applied and the ulcer healed after 24 hours. The next day it became painful and the centre had to be opened by incision. After a few days the ulcer was again treated with Parex incorporated in a mild indifferent ointment. The treatment was repeated twice a day. After three days the epithelial repair was complete and the ulceration has not returned after four months.

Other cases in which varicose ulcers and chronic tonsillitis were treated with Parex will be described elsewhere.

## SUMMARY.

Clinical trials proved Parex to be efficacious in cases of periodontal diseases, varicose ulcers and chronic tonsillitis. As these

cases have in common their being infections which show more or less putrefaction and a poor circulation, Parex will without doubt prove to be valuable in the local treatment also of other diseases. More elaborate exact indications for use will have to be worked out, starting from the principle that the remedy produces dilatation of the blood vessels, and inhibits bacterial growth and putrefaction.

## FINAL REMARKS.

Our object has been achieved: we have found the therapeutic principle of the cigar-stub and composed a medecine utilizable in practice.

The presentation of this essay aims at demonstrating the methods of argument and experiment. The report does not, however, aim at demonstrating the correctness of our initial hypothesis. When we compare the results of our experiments with the hypothesis started from, we must assume that the action of Parex is more complicated than we supposed at the outset.

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# INVESTIGATIONS ON ORGANIC FUNGICIDES

## III. THE INFLUENCE OF ESSENTIAL TRACE METALS UPON THE FUNGITOXICITY OF TETRAMETHYLTHIURAM DISULPHIDE AND 8-HYDROXYQUINOLINE [1]

by

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INTRODUCTION.

Recently, attempts have been made to relate the fungitoxicity of dithiocarbamates and of 8-hydroxyquinoline to their ability to precipitate or to form chelate complexes with various heavy metal ions. It has been demonstrated that some of these heavy metals are indispensable to fungal growth (STEINBERG (1, 2), FOSTER (3)). In the case of *Aspergillus niger* van Tieghem it has been found that essential trace elements comprise iron, zinc, copper, manganese, molybdenum and possibly gallium.

It has been suggested that the activity of the antifungal substances mentioned above may be due to precipitation or chelation of essential metallic constituents in the substrate and in the mould, this preventing growth by rendering the production of essential enzyme systems containing these elements impossible. ZENTMYER (4, 5) who was the first to propose this mechanism of action, based his theory on the results of the following experiments. He noticed that growth inhibition by 8-hydroxyquinoline of the mould *Fusarium oxysporum* var. *lycopersici* on a complete medium could be reversed by the addition of zinc ions. Further support for his theory has been furnished by ALBERT *et al.* (6) who stated that isomeric hydroxyquinolines which do not possess metal chelating properties show only weak antimicrobial activity.

ZENTMYER's precipitation theory has also been applied to elucidate the antifungal activity of dithiocarbamates (HORSFALL (7)) because of their ability to precipitate or to chelate metal ions essential to fungal growth.

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[1] Second publication: Antonie van Leeuwenhoek **16**, 282, 1950.

The mode of action of certain antibacterial substances has also been ascribed to this effect. It has been suggested by ALBERT and GOLDACRE (8) that 1-hydroxyacridine owes its antibacterial activity to its ability to combine with metabolically important trace metals. Antibacterial properties, ascribed to this metal-binding effect, have also been noted with several other heterocyclic substances bearing a hydroxylgroup *ana* to a ring-nitrogen (6).

Very recently it has been questioned whether the antimicrobial activity of chemicals such as 8-hydroxyquinoline can be completely accounted for by the precipitation theory (9, 10). Evidence against the precipitation theory is offered in the case of Cu-8-hydroxyquinolinate which has been reported to be even more active as a fungicide than 8-hydroxyquinoline itself.

MASON (9) established that not only Cu-8-hydroxyquinolinate, but also the magnesium-, iron-, zinc- and manganese compounds showed antifungal activity. The nickel-, cadmium- and silver compounds of 8-hydroxyquinoline have also been reported to be active (SEXTON (10)). ZENTMYER's finding that the toxicity of 8-hydroxyquinoline is considerably suppressed by zinc apparently contrasts herewith.

Also in our opinion criticism may be passed on the application of the precipitation hypothesis to explain the mode of antifungal action of dithiocarbamates (HORSFALL (7)). If the activity of these compounds were due to precipitation of metals essential to moulds, then it might be expected that the dithiocarbamic acid salts of these metals would be of little or no potency. However, the zinc- and iron salts of dithiocarbamic acid are powerful antifungal substances which have found wide application in the combat against various plant pathogenic fungi.

In connection with our interest in general problems concerning the mechanism of antifungal action we examined the influence of essential trace metals on the toxicity of antifungal substances more systematically. As a representative of the dithiocarbamic acid derivatives tetramethylthiuram disulphide (t.m.t.d.) was chosen. Since most experiments concerning the influence of heavy metals upon the toxicity of antimicrobial agents have been carried out with 8-hydroxyquinoline (4, 5, 6, 9, 10), this substance was involved in our investigation for purposes of comparison.

### EXPERIMENTS WITH T.M.T.D.

According to STEINBERG (1) the mould *Aspergillus niger* requires for growth the elements iron, zinc, copper, manganese, molybdenum and possibly gallium. FOSTER (3) reports that optimum concentrations of these elements for this mould are: iron 0.20 p.p.m., zinc 0.16 p.p.m., copper 0.04 p.p.m., manganese 0.02 p.p.m. and molybdenum 0.02 p.p.m.

In view of these figures *Aspergillus niger* (the mould used in our investigations) was cultivated on Czapek-Dox agar of the following composition:

| | |
|---|---|
| $H_2O$ | 1.000 ml |
| Agar [1] | 20 g |
| Saccharose | 30 g |
| $NaNO_3$ | 2 g |
| KCl | 0.5 g |
| $MgSO_4 \cdot 7\,H_2O$ | 0.5 g |
| $K_2HPO_4$ | 1 g |

to which were added the following essential trace metals (cf. FOSTER (3)):

| | | |
|---|---|---|
| $FeSO_4 \cdot 7\,H_2O$ | 1 | p.p.m. |
| $ZnSO_4 \cdot 7\,H_2O$ | 0.6 | ,, |
| $CuSO_4 \cdot 5\,H_2O$ | 0.16 | ,, |
| $MnSO_4 \cdot 7\,H_2O$ | 0.15 | ,, |
| $(NH_4)_2MoO_4$ | 0.05 | ,, |

When cultivated on this medium *Aspergillus niger* proved to be much more sensitive to t.m.t.d. than when a malt agar medium (agar 2 % + malt solution containing 10 % nonvolatile matter) or a peptone-glucose substrate (agar 2 % + peptone Witte 2 % + glucose 2 %) was used. If cultivated on a peptone-glucose substrate the mould was completely inhibited by 30 p.p.m. of t.m.t.d., whereas on malt agar medium 10 p.p.m., and on Czapek-Dox medium even 0.1 p.p.m. already proved sufficient for complete suppression of growth. Apparently, the sensitivity of *Aspergillus niger* towards t.m.t.d. highly depends on the nutrient conditions (cf. (13)).

The influence of certain trace elements on the toxicity of t.m.t.d. was investigated, cultivating *Aspergillus niger* both in a sensitive state (Czapek-Dox medium) and in a less sensitive state (malt agar medium). In the experimental procedure the concentration of one trace element was varied, whereas, as far as Czapek-Dox agar was concerned, all other trace elements were added to the agar in the quantities mentioned. In the case of malt agar an additional supply of trace elements was considered superfluous.

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[1] The agar was first thoroughly rinsed with streaming tapwater for 3 days and then repeatedly treated with distilled water during 4 days.

The sensitivity of the mould towards t.m.t.d. was determined in a graded series of concentrations. Dilutions of the antifungal substance under investigation were prepared in acetone, and 0.1 ml of each dilution was pipetted into tubes containing 10 ml of melted agar maintained at 50°C. in a water bath. After adding the trace metal salts (dissolved in water) the agar was thoroughly mixed and then poured into Petri dishes of 8 cm diameter. After solidification the plates were streaked with a suspension of spores of *Aspergillus niger* and subsequently incubated at 30° C. Readings could be taken after two days. The small amount of acetone in the medium had no demonstrable inhibitory effect on mould growth.

The concentration series of the metals used were:

0, 1, 5, 20 and 100 p.p.m. $FeSO_4 . 7 H_2O$;
0, 0.5, 2, 10 and 50 p.p.m. $ZnSO_4 . 7 H_2O$;
0, 0.1, 0.5, 2, 10 and 50 p.p.m. $CuSO_4 . 5 H_2O$;
0, 0.1, 0.5, 2, 10 and 50 p.p.m. $MnSO_4 . 7H_2O$;
0, 0.05, 0.2, 1, 5, 20 and 100 p.p.m. $(NH_4)_2MoO_4$.

The concentration series of t.m.t.d. was:
0.025, 0.1, 0.5, 2.5, 10 and 50 p.p.m.

In these experiments it was found that none of the metals in the concentrations employed had any perceptible influence upon the toxicity of t.m.t.d. On the Czapek-Dox medium in all cases 0.1 p.p.m. of the antifungal agent produced a complete inhibition of fungal growth, whereas on the malt medium invariably 10 p.p.m. were required.

## EXPERIMENTS WITH 8-HYDROXYQUINOLINE.

Like in the case of t.m.t.d., the sensitivity of *Aspergillus niger* towards 8-hydroxyquinoline proved to be highly dependent on the composition of the substrate. If cultivated on Czapek-Dox agar the mould was completely inhibited by 0.1 p.p.m. of 8-hydroxyquinoline, whereas in the case of malt agar 10 p.p.m. were required.

The experimental procedure was the same as in the experiments with t.m.t.d. The metals zinc, copper, iron, manganese and molybdenum, together with the antifungal agent 8-hydroxyquinoline were applied in the same dilution series as was used previously. In addition, the influence of cobalt was investigated because this metal, although not known to be essential to moulds, has been reported to exhibit a neutralizing effect on the toxicity of the substance under consideration in the case of various other micro-organisms (6).

It was found that addition of zinc, copper, manganese and molybdenum salts in the concentration employed did not reverse the toxicity of 8-hydroxyquinoline, regardless whether Czapek-Dox agar or malt agar was concerned.

Addition of iron ions, however, markedly reduced the antifungal activity of the substance under study in the case of malt agar (cf. table I and plate 1). In the case of Czapek-Dox agar, upon which the mould was found to be already completely inhibited by 0.1 p.p.m. of 8-hydroxyquinoline, addition of iron ions did not counteract the chemical (cf. table II).

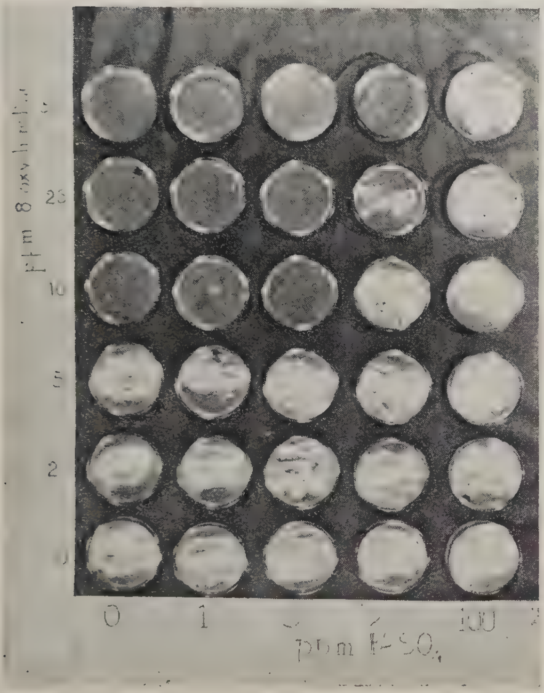
TABLE I.

Growth of *Aspergillus niger* on malt agar; the toxicity of 8-hydroxyquinoline is reversed by addition of iron ions.

| $FeSO_4 . 7 H_2O$ | 0 p.p.m. | 1 p.p.m. | 5 p.p.m. | 20 p.p.m. | 100 p.p.m. |
|---|---|---|---|---|---|
| blanc | +++ | +++ | +++ | +++ | +++ |
| 8-hydroxyquinoline | | | | | |
| 2.5 p.p.m. | +++ | +++ | +++ | +++ | +++ |
| 5 ,, | +++ | +++ | +++ | +++ | +++ |
| 10 ,, | — | — | — | +++ | +++ |
| 25 ,, | — | — | — | + | +++ |
| 50 ,, | — | — | — | — | ++ |

In the experiments with cobalt ($Co(NO_3)_2$) a somewhat different effect was noted. Like iron, this metal reduced the toxicity of 8-hydroxyquinoline under conditions at which the test mould showed a low sensitivity to the antifungal substance (on malt agar) but in the case of a high sensitivity (on Czapek-Dox agar) a slight reversal was noted as well. The results of the experiments with this metal are given in table III and IV.

## PLATE 1.



The influence of increasing amounts of $FeSO_4$ on the antifungal action of 8-hydroxyquinoline against *Aspergillus niger* cultivated on malt agar.

## TABLE II.

Growth of *Aspergillus niger* on Czapek-Dox agar; the toxicity of 8-hydroxyquinoline is not reversed by addition of iron ions.

| $FeSO_4 . 7 H_2O$ | 0 p.p m. | 1 p.p.m. | 5 p p m. | 20 p.p.m. | 100 p.p.m. |
|---|---|---|---|---|---|
| blanc | ++ | ++ | ++ | ++ | ++ |
| 8-hydroxyquinoline | | | | | |
| 0.1 p.p.m. | — | — | — | — | — |
| 0.5 ,, | — | — | — | — | — |
| 2.5 ,, | — | — | — | — | — |

TABLE III.

Reverse of toxicity of 8-hydroxyquinoline to the mould *Aspergillus niger* by addition of cobalt ions to a malt agar medium.

| $Co(NO_3)_2$ | 0 p.p.m. | 1 p.p.m. | 5 p.p.m. | 20 p.p.m. | 100 p.p.m. |
|---|---|---|---|---|---|
| blanc | +++ | +++ | +++ | +++ | +++ |
| 8-hydroxyquinoline | | | | | |
| 2.5 p.p m. | +++ | +++ | +++ | +++ | +++ |
| 5 ,, | ++ | +++ | ++ | +++ | + |
| 10 ,, | — | — | ++ | ++ | + |
| 25 ,, | — | — | — | ++ | + |
| 50 ,, | — | — | — | — | + |

TABLE IV.

Reverse of toxicity of 8-hydroxyquinoline to the mould *Aspergillus niger* by addition of cobalt ions to a Czapek-Dox agar medium.

| $Co(NO_3)_2$ | 0 p.p.m. | 1 p.p.m. | 5 p.p.m. | 20 p.p.m. | 100 p.p m. |
|---|---|---|---|---|---|
| blanc | ++ | ++ | ++ | ++ | ++ |
| 8-hydroxyquinoline | | | | | |
| 0.1 p.p.m. | ± | ± | + | + | ++ |
| 0.5 ,, | — | — | — | — | — |
| 2.5 ,, | — | — | — | — | — |

## DISCUSSION OF RESULTS.

The antifungal activity of chemicals is governed by two factors which may be designated as inherent toxicity and availability(7, 12).

Apart from factors such as permeation, availability of a toxicant is primarily connected with its solubility. A substance of high inherent toxicity may be limited in its potency by its low solubility in water, so that the concentration causing a complete inhibition of growth is not reached. In this case the chemical may be considered unavailable to the organism. It may thus occur that the availability of an agent is such that organisms which show a high sensitivity to the agent may be completely suppressed, whereas a slightly less sensitive organism may not be suppressed at all.

Taking these considerations into account most of the results of the present experiments as well as some of those obtained by earlier investigators in this field can be explained.

The precipitation theory suggests that the anti-microbial activity of certain agents is attributable to their ability of combining with certain trace elements, rendering the latter unavailable as essentialities for microbial growth. This implies, reversely, that addition of a sufficient quantity of a heavy metal renders the chemical unavailable. Evidence in favour of the precipitation theory has been furnished by ZENTMYER (4, 5) in experiments in which a zinc salt and the antifungal agent 8-hydroxyquinoline were combined. Evidence against the precipitation theory has been brought forward by MASON (9) and by SEXTON (10) who reported several heavy metal salts of 8-hydroxyquinoline, among which the zinc salt, to be quite antifungal. This is in accordance with the results of one of our experiments in which addition of zinc was found not to reverse the toxicity of 8-hydroxyquinoline to any perceptible degree.

An explanation of this discrepancy may be found in the following. Working with a *Fusarium* species which had been grown on a peptone-maltose medium, ZENTMYER found inhibition of growth at 40 p.p.m. of 8-hydroxyquinoline. Under the circumstances of our experiments the test mould used (*Aspergillus niger*) was found to be more sensitive to this chemical, on a malt agar medium 10 p.p.m., and on Czapek-Dox medium only 0.1 p.p.m. of the substance being sufficient for complete inhibition. Apparently, the solubility of the zinc salt was sufficient to reach the toxic level under the growth conditions in our experiments, whereas in ZENTMYER's investigations this was not the case.

The results obtained in our experiments with iron may be explained in a similar way. This metal was found to reverse the agent under study only in the case of malt agar (cf. table I and plate 1), whereas on a synthetic medium no evidence for antagonism was found. Apparently, the solubility (in malt agar) of Fe-8-hydroxyquinolinate formed must be accepted insufficient to reach the concentration required for a complete suppression of *Aspergillus niger*. However, using Czapek-Dox agar (the sensitivity of the mould to the agent being a hundred times higher) the solubility of the iron compound must have been sufficient to achieve complete growth inhibition.

This conception is supported by an experiment with a laboratory preparation of the iron salt of 8-hydroxyquinoline. This compound was found to possess a high toxicity against *Aspergillus niger* when cultivated on Czapek-Dox agar, whereas on malt agar the substance proved to be completely inactive.

In table V the activities against *Aspergillus niger* of 8-hydroxyquinoline and its Fe-salt under the growth conditions mentioned are compared.

TABLE V.

Comparison of the activities of 8-hydroxyquinoline and Fe-8-hydroxyquinolinate against *Aspergillus niger* cultivated on Czapek-Dox agar and malt agar respectively.

| | Concentrations causing complete inhibition of growth | |
|---|---|---|
| | Czapek-Dox agar | Malt agar |
| 8-hydroxyquinoline | 0.1 p.p.m. | 10 p.p.m. |
| Fe-8-hydroxyquinolinate | 0 2 p.p.m. | inactive |

If the toxicity of 8-hydroxyquinoline were due to combination with iron ions it might be expected that the combination product (the iron salt) would be non-toxic.

However, the figures given in column 1 of table V show that, when compared on a molecular basis, the iron salt of 8-hydroxyquinoline has about the same inherent toxicity as free 8-hydroxyquinoline. It thus follows that the fungitoxicity of 8-hydroxyquinoline cannot be attributed to its ability to combine with iron ions.

The solubility of the iron salt apparently is insufficient to reach the concentration required to inhibit the growth of *Aspergillus niger* when cultivated on malt agar. In other words, the iron salt is ineffective here not because it is inactive but because its effective concentration is not reached.

The same conclusions hold *a fortiori* for the remaining essential heavy metals, because here an antagonistic effect was not observed even in the case of malt agar.

The inhibitory action of 8-hydroxyquinoline on bacteria has been ascribed to its ability to remove by chelation the heavy metal cobalt which has been demonstrated to be a vitally important element for several bacteria. Although this element has never been reported essential to moulds, it seemed worthwhile to include some experiments with this metal.

In the addition of cobalt to a malt agar medium the same phenomena were encountered as with iron. However, cobalt appeared to be also antagonistic to the action of 8-hydroxyquinoline when applied to a Czapek-Dox medium (cf. table IV), but in this case the antagonism was very weak. As many as 100 p.p.m. of $Co(NO_3)_2$ were required to nullify the effect of 0.1 p.p.m. of 8-hydroxyquinoline while with 0.5 p.p.m. of the latter agent complete inhibition was again effected.

An interesting property of cobalt has been reported by ALBERT and co-workers (6, 8). These investigators noticed a sharp difference in sensitivity towards 8-hydroxyquinoline between Gram-positive and Gram-negative bacteria, the former being considerably more sensitive than the latter. In the presence of an inhibitory concentration of 8-hydroxyquinoline only cobalt appeared to be effective in restoring growth of Gram-positive bacteria, whereas in the case of Gram-negative species not only cobalt but also zinc, iron and copper effected reversal.

This difference in behaviour towards heavy metals between the two classes of bacteria is highly reminiscent of the observation with the mould *Aspergillus niger* described in the present investigation. As already noted, the action of 8-hydroxyquinoline upon *Aspergillus niger* cultivated in a sensitive state is exclusively reversed by Co ions. However, if the mould is cultivated in an insensitive state the toxicity of 8-hydroxyquinoline is also reversed by other heavy metals.

This suggests that the observed differences in behaviour towards heavy metals between Gram-negative and Gram-positive bacteria in the presence of 8-hydroxyquinoline seem to be merely a consequence of differences in availability of 8-hydroxyquinoline. Consequently, these differences can not be regarded significant as a fundamental distinction between the two classes of bacteria.

In our opinion the explanation of most phenomena concerning the influence of heavy metal ions on the activity of 8-hydroxyquinoline must be sought in the availability factor. There are no obvious reasons to accept the metal-precipitating properties of 8-hydroxyquinoline as the origin of its antimicrobial action.

Also in the case of t.m.t.d. there seems to be little evidence for an explanation of the mechanism of action in the sense of the precipitation theory.

## Summary.

The influence of the trace metals Fe, Zn, Cu, Mn and Mo — known to be essential for the growth of the mould *Aspergillus niger* van Tieghem — upon the fungitoxicity of tetramethylthiuram disulphide (t.m.t.d.) and of 8-hydroxyquinoline was investigated using the same mould as the test organism. It was found that none of the metals upon addition to the medium reversed the toxicity of t.m.t.d. to any perceptible degree neither when the mould was cultivated in a state of being very sensitive to t.m.t.d. (on a synthetic medium) nor when it was grown in a state of being about a 100 times more resistant to the agent (on a malt agar medium).

Addition of Zn, Cu, Mn and Mo did not counteract the antifungal activity of 8-hydroxyquinoline. Fe effected a reverse only when the mould was cultivated in a resistant state, whereas this metal had no influence upon the toxicity of 8-hydroxyquinoline when the mould was cultivated in a sensitive state. The iron salt of 8-hydroxyquinoline showed about the same antifungal activity as free 8-hydroxyquinoline only when the test mould was cultivated under conditions favouring a high sensitivity.

Most phenomena encountered in the reversal of toxic action of antimicrobial agents by addition of heavy metal ions are satisfactorily explained by the ,,availability'' factor.

It was concluded that the antifungal activity of tetramethylthiuram disulphide and 8-hydroxyquinoline cannot be attributed to their capability of precipitating essential heavy metals.

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# SOME THEORETICAL ASPECTS OF SYMBIOSIS ¹)

by

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## 1. The concept „symbiosis".

The concept „symbiosis" was used for the first time by the Swiss botanist de Bary in 1878. He defined symbiosis as: „Die Erscheinungen des Zusammenlebens ungleichnamiger Organismen". This „living together" can be of all kinds. De Bary himself already distinguished between mutualistic symbioses where both organisms profit by the presence of the partner and antagonistic symbioses where at least one of the partners is injured by the other one. Perhaps this classification can be somewhat extended by restricting the word antagonistic to those „symbioses" where both organisms are injured by the presence of the other one, whereas we speak of parasitic „symbioses" when one of the partners is injured by the association while the other profits by it.

Mostly, however, the word symbiosis has been restricted to the mutualistic symbioses (symbiosis s.s.). It cannot be denied that the mutual benefit as presupposed for a mutualistic symbiosis for most symbioses has been more or less expected or even desired while the experimental data could produce little evidence for this supposition. So when certain symbioses were studied more carefully the presupposed benefit appeared to be rather doubtful. Too many biologists had failed to realize that two organisms don't enter into a

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¹) The main part of this article consists of a lecture delivered at a symposium on adaptation during the XXXIth "Nederlands Natuur- en Geneeskundig Congres" in Groningen April 20, 1949. After a request of one of the editors to publish this lecture in Antonie van Leeuwenhoek, the lecture has been somewhat changed and extended to make it more suitable for publication in this journal.

symbiosis to give something to the partner, but in order to take as much advantage of the partner as possible. It appeared that the partners in a mutualistic symbiosis try to take advantage of each other in a way which does not at all differ from the behaviour of a parasite towards its host. This may account for the continual conflict between those investigators who understand by a certain biological association a (mutualistic) symbiosis and those who understand by the same association a parasitism.

The cases of symbiosis may be distinguished as conjunctive symbioses where the two organisms are in direct contact with each other and disjunctive symbioses where the two organisms influence each other at some distance. While the question arises whether in nature truly mutualistic conjunctive symbioses do indeed exist, among the disjunctive associations truly mutualistic symbioses may be found. In this respect the artificial symbioses of SCHOPFER (8) must be mentioned where two organisms which when alone can only grow after addition of a nutrilite can, when together, grow without the addition of their respective nutrilites because each of them synthesizes the nutrilite necessary for the growth of the partner. There is no doubt that analogous cases of symbiosis are very common in nature. Furthermore I point to the investigations on antibiotics for examples of truly antagonistic ,,symbioses''. So while some disjunctive symbioses may be called truly mutualistic or truly antagonistic, it is much more difficult to determine the nature of most conjunctive symbioses (which of course are never truly antagonistic). These associations are so very complex that only a careful analysis of the individual relations between the two organisms enables us to class them with the mutualistic-, or parasitic symbioses. We shall consider some details of these relations.

When two organisms come into each other's sphere of influence many influences will be exercised. We can distinguish two groups of influences: influences which favour the partner in any way (symbiotic influences) and influences which are unfavourable to the partner (antibiotic influences). These symbiotic and antibiotic influences may be quite different in character and when I try to take them together in a short survey I am fully aware of the fact that the groupings of this survey cannot be sharply distinguished from each other nor do I pretend that all influences actually exercised will come under one of the groupings of the survey. I only intend to give a conveniently arranged summary of the most important influences which two organisms may have upon one another in a symbiosis (whether conjunctive or disjunctive, whether mutualistic, parasitic or antagonistic). As I want to restrict myself to the physiological symbioses, the psychological or ethological influences which play a part in many zoological symbioses are left out of consideration.

Symbiotic influences.

1. Environment. A symbiotic influence may consist of a change of the environment so that it becomes more suitable for the development of the partner. E.g. an anaerobic organism will be influenced symbiotically by an aerobic organism which absorbs the injurious oxygen or lowers the redox-potential in any other way. Comparable symbiotic influences consist of a changing of the pH, of the ion-relations, of the conditions of light and so on. In the conjunctive symbioses this means that one of the organisms creates in or on its cells a milieu where the partner can develop pre-eminently. Mostly these kinds of influences are rather unspecific and the question arises whether associations where only such influences play a part (epiphytism, endophytism) deserve the name of symbiosis.

2. Food. One of the organisms may synthesize some food substances which are used by the partner as a source of energy. E.g. the autotrophic alga in a lichen synthesizes carbohydrates which are utilized by the heterotrophic fungus. It may be that a compound which is already present in the medium is made available for the enzyme-systems of one organism by the hydrolytic enzymes of another as is the case with cellulase-production in the soil.

3. Nutrilites. It has been shown that in nature spontaneous mutants may arise which have lost the capacity of synthesizing certain essential cell-constituents. These organisms can only develop when the lacking cell-constituents (e.g. co-enzymes, amino acids, nucleic acids) are present as such in the medium (nutrilites). Then another organism can exercise an important symbiotic influence upon this mutant, if it has not lost the capacity of synthesizing this nutrilite so that the mutant can make use of it. These kinds of symbiotic influences can be very specific and it is especially the need for certain nutrilites which may explain an obligatory dependence of one organism on another.

Antibiotic influences.

1. Environment. One of the organisms can inhibit the development of the partner by changing the environment in an unfavourable way. When an aerobic organism absorbs oxygen and lowers the redox-potential the development of other obligatory aerobic organisms is made impossible; an organism which produces acids impedes the development of those organisms which only thrive at a higher pH and so on. In this respect the ecological factor ,,space'' must be mentioned, e.g. a plant can prevent the further development of a parasitic fungus by forming suberized cell-layers which cannot be penetrated by this fungus.

2. Food-competition. Food-competition plays a part wherever the amount of available food-substances is a limiting factor. E.g. in a lichen the fact that the fungus withdraws organic substances from the algae may be considered as an antibiotic influence upon the algae.

3. Antibiotic substances. The most specific influence which one organism may exercise upon another is the production of specifically toxic substances. These are well-known among disjunctive symbioses (antibiotics like penicillin, gramicidin, streptomycin etc.), though among conjunctive parasitisms their influence has been observed as well (e.g. lycopersin in the parasitism of *Fusarium* on tomato plants: Gäumann and Jaag (4)). In the conjunctive biological associations substances with a higher molecular weight may play a still more important part (lytic enzymes, antigens and so on).

So when two organisms come into each other's sphere of influence one or more of these symbiotic or antibiotic influences may be active between them. In an absolute antagonism only antibiotic influences from both sides will occur, in an absolute mutualism only symbiotic influences from both sides will be found while in an absolute parasitism only symbiotic influences from one side, only antibiotic influences from the other side are exercised. This, however, is an exception. In nearly all cases symbiotic as well as antibiotic influences are exercised from both sides and it will only depend on the mutual relation between these whether the association as a whole has to be classed with the antagonisms, the mutualisms or the parasitisms. In an antagonism from both sides the antibiotic influences predominate over the symbiotic influences, in a mutualism the reverse happens, in a parasitism from one side the symbiotic influences, from the other side the antibiotic influences prevail. This simultaneous occurrence of symbiotic and antibiotic relations had to be emphasised as it accounts for the ever returning controversy as to the mutualistic or parasitic character of a certain association, where often one investigator considers the symbiotic relations only, while another considers the antibiotic relations in a too one-sided manner.

So the limits between antagonism, parasitism and mutualism are more gradual than real and this is the more striking if we consider that the occurrence of symbiotic or antibiotic influences is not a constant peculiarity of the two organisms in question, but that this occurrence is in many cases dependent on internal (age) or external (environment) factors. This explains perhaps why many associations at one moment give the impression of a perfect mutualism while at another moment the same association has the character of a mercyless parasitism!

As it appears impossible to make a clear distinction between mutualism, parasitism or even antagonism the restriction of the concept ,,symbiosis'' to the first of these groups loses much of its original significance. But even when using the concept ,,symbiosis'' in the original definition of DE BARY the question arises whether or not the relationships between organisms of different names are so essentially different from the relations between organisms of the same name as to justify us in restricting the word symbiosis to the former associations. An artificial symbiosis like the one described by SCHOPFER between *Mucor Ramannianus* and *Rhodotorula rubra* is of exactly the same nature as a symbiosis between two mutants of *Neurospora* (BEADLE (1)). Between organisms belonging to the same species e.g. in cultures of yeast or bacteria symbiotic and antibiotic relations are found which may be compared in any respect to the symbiotic and antibiotic relations described above. Every microbiologist is familiar with the phenomenon that in certain media some organisms only develop after heavy inoculation. This phenomenon has been explained by the assumption of some change in the environment (e.g. the redox-potential) or by the production of nutrilites (bios-experiment of WILDIERS). But also antibiotic influences are known to occur between organisms belonging to the same species. In colonies food-competition may play an important part and even the production of antibiotics has been observed (chlorellin in cultures of *Chlorella* (5, 6)). The first biologist we know who turned his attention to the similarity between associations of organisms of different names and associations of organisms of the same name was Baas Becking (unpublished, first mentioned by Quispel (7)), who distinguished between homosymbiosis and heterosymbiosis. He even went a step further by describing the associations of cells and tissues in higher organisms as histosymbiosis. In 1947 Cowdry (2) arrived at the same conclusions and gave some striking examples of typical symbiotic and antibiotic relations between cells and tissues of the human body. For examples of symbiotic and antibiotic relations between cells of plant tissues I point to the part played by auxins and vitamins during growth and of inhibiting substances during ripening of seeds.

The concept ,,symbiosis'' may be extended in yet another direction. The association between two mutants of *Neurospora* which can grow together without the addition of nutrilites because of the fact that each of them synthesizes the nutrilite required by the other will be considered by everybody as a symbiosis. Beadle (1) succeeded in obtaining a dikaryontic mycelium from two such mutants. This dikaryontic mycelium was autotrophic with regard to the addition of nutrilites though it was known that each of the nuclei lacked the capacity to synthesize a certain nutrilite. Beadle describes this mycelium as a internuclear symbiosis. When these two nuclei fuse a diploid nucleus is formed. Though again this diploid mycelium is autotrophic with respect to nutrilites we know that both parts of the nuclei lack the ability to synthesize a nutrilite. Beadle describes this mycelium as an intranuclear symbiosis. If we try to be consistent we must extend the concept ,,symbiosis'' to the co-operation of genes. It would be tempting to describe certain genetical processes in terms of symbiotic and antibiotic relations between genes! Just as an isolated virus particle has lost the capacity of multiplying and only multiplies when influenced by the host (symbiotically!) an isolated gen will be nothing more than a chemical substance which can only unfold its activities under the symbiotic influences of other cell-constituents.

Thus we can apply the concepts ,,symbiosis'' and ,,antibiosis'' to all integration-levels: species, organisms, organs, tissues, cells, nuclei, and genes. Because of the latter views the question arises whether the concepts ,,symbiosis'' and ,,antibiosis'' have to be considered as covering the most central problem of biology. The late H. J. JORDAN once said: ,,Das Problem der ganzen Biologie ist die Organisation oder die Koordination''. The problem of coordination is nothing but the problem of symbiosis and antibiosis in the widest sense of these terms.

The extension of the concept ,,symbiosis'' has this drawback: The concept ,,symbiosis'' loses much of its original significance as ultimately the whole field of biology can be described in terms of symbiosis and antibiosis. As a matter of fact most biologists will want to restrict the concept ,,symbiosis'' more or less to the original definition of DE BARY. Then, however, we must realize that we isolate a group of phenomena from other groups of closely related ones in a more or less subjective manner.

## II. THE EVOLUTION OF SYMBIOSES.

When we return for a moment to the old concept of DE BARY we can ask the question why two organisms entered into a certain association. The answer to this can be short. An organism enters into a biological association with another organism because it receives some symbiotic influences from this other organism. E.g. in the neighbourhood of the other organism better ecological conditions are found, necessary food-substances or nutrilites can be obtained which are less easily found outside the sphere of influence of the partner. Moreover, it is necessary that no antibiotic influences interfere with the association. In phytopathology many examples are known of parasites which are specialized on one host-plant only. However, they can be cultivated upon extracts of many plants so that these plants can certainly provide all food substances and nutrilites. Yet one plant only can be attacked in nature, most probably because of the absence of antibiotic influences in the widest sense of this term (GÄUMANN (3)).

When these requirements are met an organism fits the association. In an antagonism none of the partners fits the association, in a parasitism only one of the partners fits it, in a mutualism both partners fit it. As we have seen that it is impossible to make a clear distinction between antagonism, parasitism and mutualism we must expect that different degrees of ,,fitting'' occur. Generally speaking an organism fits an association the better when it receives from and exerts upon the partner more symbiotic and fewer antibiotic influences. When only few symbiotic influences occur in an association the partners will be dependent on each other in few respects and the association will mostly have a facultative character. When more symbiotic relations are found the partners will be more dependent on each other and the association may obtain an obligatory character.

When an organism has entered into a certain association with another organism because of one of the reasons mentioned above an adaptation may develop in consequence of which the organism will fit the association better. This adaptation may differ in nature.

It may be that within the possibilities of the variability of the species certain changes occur in an individual or in a group of individuals. A good example of this ,,phaenotypical'' adaptation is given by the so called enzymatic adaptation: the production of enzymes only when the specific substrate is present. It may be possible that an organism which entered an association with another organism begins to produce some enzymes which hydrolyze the reserve substances of the partner by means of the process of enzymatic adaptation. Another example of phaenotypical adaptation is given by the adaptation of a parasite to a new host, the loss of parasitic virulence after saprophytic cultivation and the regaining of virulence after some passages through a host. These phaenotypical adaptations are very important in facultative associations where a symbiotic or parasitic mode of life alternates with periods of saprophytism or where different organisms can be used as partners, so that after entering the new association the partners have to adapt themselves to the new circumstances. In obligatory associations the importance of these kinds of adaptations is much smaller.

When considering the evolution of symbiosis the genotypical adaptations are much more important. Without resorting to accepting neo-Lamarckian theories we can assume that by means of natural selection of spontaneous mutants genotypical changes of the partners may occur which result in a better adaptation to the association. After this adaptation the partners fit the association better than before. We want to elucidate this genotypical adaptation by two examples.

1. It is a well-known fact that in cultures of micro-organisms mutants may appear of which one gen has changed in such a manner that a certain important substance cannot be synthesized by the mutant. Then this substance has to be present as a nutrilite in the medium. In nature the possibility exists that such mutants cannot

survive so that they are selected away. When, however, such mutants appear in organisms symbiotically living there will be no difference between the viability of the original organisms and the mutants because the lacking substance can be obtained from the partner in the association. So the percentage of such mutants will be relatively greater among organisms living in associations with other organisms than among their relatives that live freely. Consequently owing to the survival of these mutants in biological association the importance of symbiotic influences will increase.

2. It is a well-known fact that some strains of bacteria are resistant to the action of certain antibiotics. Under the influence of these antibiotics all other strains will be killed or injured so that the resistant strain is selected out. We may expect that the same thing will happen when strains of an organism are resistant to the action of the antibiotics (in the widest sense of the term) produced by the partner in a biological association. Here resistant strains will be selected out too, so that the relative percentage of such strains will increase and the importance of antibiotic influences decreases.

These two selection mechanisms have the following results:

1. By the relative increase of the symbiotic influences the partners of an association will become more and more dependent on each other. Consequently an association which in the beginning was facultative becomes more and more obligatory (e.g. epiphytism — facultative parasitism — obligatory parasitism).

2. The relative increase of the symbiotic influences and the relative decrease of the antibiotic influences even has another and more important result: the association which in the beginning was antagonistic or parasitistic becomes more and more mutualistic. If this is true we must expect that the mutualisms belong to the biological associations that are oldest phylogenetically, an expectation which is also suggested by other observations.

The conclusion might be drawn that at the end of this genotypical adaptation the ideal mutualism is reached in which only mutualistic influences are exercised between the partners and all antibiotic influences are absent. This, however, is not the case.

The fact is that influences which are antibiotic for one of the partners are in many cases symbiotic for the other and inversely. When the fungus of a lichen takes carbohydrates from the alga the latter is deprived of its reserve-substances. The symbiotic influenc upon the fungus is at the same moment an antibiotic influence upon the alga. When in a mycorrhiza the plant produces lytic enzymes which locally destroy the cells of the fungus this must be considered as an antibiotic influence upon the fungus. As to the plant, this production of lytic enzymes is coupled with a very important symbiotic influence from the fungus: by this lytic activity nitrogenous substances and most probably nutrilites as well get at the disposal of the plant.

We shall dwell at some length on the latter example. Let us suppose that a strain of the fungus appears which is resistant to the action of these lytic enzymes. Then, originally, this fungus will fit the association better than the non-resistant fungi. The consequence is, however, that a very important symbiotic influence from the fungus upon the plant fails to appear so that the plant will be impaired. This will have its reaction upon the fungus, which e.g. may get fewer carbohydrates from the weakened plant. So, ultimately, the resistant fungus will be in a not at all better situation as compared with its non-resistant relatives. The end of the adaptation-process here will not be the ,,ideal'' mutualism without any antibiotic influences but a very special equilibrium between the mutual symbiotic and antibiotic influences in which of course the symbiotic influences will be in the majority but where so many antibiotic influences are active as is necessary to benefit the association as a whole. Expressed in other terms: while originally the genotypical adaptation of the partners of an association is ,,directed'' towards the benefit of the individual partners, ultimately, when the association has reached the stage of a perfect obligatory mutualism the adaptation can only be "directed" towards the benefit of the association as a whole. There was some reason for tempting the well-known lichenologist TOBLER (9, 10) to make the nearly classical statement: ,,Die duale Natur ist heute nicht mehr das Wesen der Flechten, nein ihr Wesen ist die neue Einheit!''

It would be interesting to consider the question whether the same reasoning is applicable to the concepts of symbiosis proposed by BAAS BECKING, COWDRY and BEADLE.

We saw above that the relations between organisms of the same name are comparable with the relations between organisms of different names. Among unicellular algae many species form colonies (or homosymbioses in the terminology of BAAS BECKING) whilst closely related species remain unicellular. It would be very interesting to trace whether this is coupled with a predominance of (homo) symbiotic influences in the colonial algae and a predominance of (homo)antibiotic influences in the unicellular algae. As far as we know an investigation into this question has never been made. For the time being it is a mere hypothesis to assume that certain (homo)symbiotic influences cause the colonial algae to remain together. It is probable that as soon as a colony has been formed a similar adaptation process will occur as was described for the heterosymbioses resulting in an increasing importance of the symbiotic influences and a decreasing importance of the antibiotic influences, there being ultimately a very special equilibrium between the symbiotic and antibiotic influences in which the symbiotic influences predominate and the antibiotic influences are only present as far as their action is necessary to benefit the colony as a whole.

If we can consider the formation of colonies in unicellular organisms as the first step in the evolution towards multicellular organisms we may expect that in multicellular organisms (the histosymbiosis in the terminology of BAAS BECKING) this equilibrium between the (histo)symbiotic and the (histo)antibiotic influences has been reached. Here we do observe that the antibiotic influences from some cells or tissues upon other cells or tissues are detrimental to the latter but are definitely favourable to or even indispensable for the organism (the histosymbiosis) as a whole.

The question whether a similar adaptation has occurred during the evolution of inter- and intranuclear symbioses must remain unanswered. Though this question is related to the most fundamental problems of biology and evolution it would lead us too far upon the path of an hypothesis that cannot be proved.

## Conclusion.

Our conclusion may be that a symbiosis may come into existence because the absence of antibiotic influences makes it possible and the presence of one or more symbiotic influences makes it necessary. Once a symbiosis has been formed a genotypical adaptation by means of natural selection of spontaneous mutants occurs. Because of this adaptation the association becomes obligatory and an ideal mutualism is reached in which only so many antibiotic influences are active as is necessary to benefit the association as a whole.

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(From the National Health Institute, Utrecht).

# *SALMONELLA BOVIS MORBIFICANS* IN MAN AND ANIMAL

by

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## CLINICAL OBSERVATION.

M. de W. the second female child of a healthy father and a mother suffering from acute endometritis presented in the age of 9 days a left sided pneumonia followed by empyema, cured by drainage and penicillin.

At the age of 6 months the baby was interned in the hospital with mucosanguinolent motions, exsiccosis and toxicosis. This disease begun as an acute gastroenteritis. Blood examination showed 10100 leucocytes with 4 eosinophiles, 0 basophiles, 6 juveniles, 14 polynuclears, 72 lymphocytes, 4 monocytes; and distinct toxic granulations. The sedimentation rate was 7/II. The feces was examined in the National Health Institute (N.H.I.) and *Salmonella bovis morbificans* could be cultivated.

The treatment consisted in starvation during 24 hours, infusion with saline and glucose, sulfoguanidin orally 1 g in 24 hours. Afterwards the diet consisted in fat-poor protein milk gradually changed against protein milk. After 10 days the baby was clinically cured: no germs could be cultivated from the feces.

Ten days after cure we sent serum to the N.H.I. in order to study agglutination with *Salmonella bovis morbificans*. The patient's strain was lost but with a laboratory one the titer was:

| | |
|---|---|
| 0 VI, VIII | 1 : 40 pos. |
| r | 1 : 40 pos. |
| 1,5 | neg. |

We examined serum of 8 healthy men in order to see if our titers might have clinical value. The result was:

0 VI, VIII 1 : 20 neg. in all cases
r 1 : 20 pos. in only one case
1,5 1 : 40 pos. in only one case

Compared with the patient's serum we interpreted the titer as the proof of a cured infection.

In the same dwelling as our little patient lives an old crippled bedridden woman of 65: she suffered some weeks from a long lasting diarrhoea with mucosanguinolent motions. When she seemed cured the baby fell ill. We sent serum and feces of this woman to the N.H.I. and according to our expectation no *Salmonella* could be cultivated from the stool. Examination of the serum however gave the following result:

0 VI, VIII 1 : 40 pos.
r 1 : 160 pos.
1,5 1 : 20 neg.

In this case we had to accept the diagnosis of a cured Salmonellosis bovis morbificans. Our attempts to find out the source of the infection remained without success: there was not the least indication of food intoxication, no contact with mice, rats, cats or dogs.

### Survey of literature.

*Salmonella bovis morbificans* is a representant of the C group with the antigen formula VI, VIII : r-1,5. This strain has been rarely found and causes an acute gastroenteritis. The first description of *S. bovis morbificans* is from Basenau (1893) who cultivated this germ from the meat of a cow slaughtered on account of septic puerperal metritis. Bruce and White (1926) studied the composition of antigens carefully. Sladen and Scott (1926) were the first to demonstrate this *Salmonella* strain as the cause of gastroenteritis in man. After consumption of pressed beef in Swansea 39 persons fell ill. From 2 patients serum had been examined for agglutination against *S. bovis morbificans*: 9 and 16 days after the beginning of the disease titers varied from 1/60 to 1/200. It was impossible to find out the way in which the meat could have been contaminated.

In Kauffmann's: "Die Bakteriologie der *Salmonella* Gruppe" (2) several food poisonings are mentioned, f.i. after consumption of horse meat. The given historiae morbi lack any indication of the source of contamination.

BRUNE and JOYCE (1947) studied in Italy during the Second World War 1789 cultures of *Salmonella*: only two belonged to *Salmonella bovis morbificans*. Between 1934 and 1947 EDWARDS, BRUNER and MORAN (1) examined 12331 cultures of *Salmonella* originating from man and animal: 6 strains belonged to *S. bovis morbificans*. Five strains were from human origin i.e. 3 patients suffered from acute gastroenteritis and 2 cases appeared to be latent carriers and one strain was from a not identified rodent.

SELIGMANN, SAPHRA and WASSERMANN (3) studied an epidemic amidst nurslings in a hospital. In the Salmonella Center in New-York *S. bovis morbificans* was studied for the first time in 1945.

Food poisoning by *Salmonella* in Danmark (1936—1940) was caused by *S. bovis morbificans* in 2 cases out of 375, in Germany (1928—1933) in 17 out of 883 and in England in 7 out of 374 cases.

In the Netherlands no further publications about new cases of *S. bovis morbificans* have appeared after BASENAU. During the last two years 8 cases of human infections were found in the N.H.I. in Utrecht probably from patients suffering from serious enteritis but clinical and epidemiological facts are lacking in 7 cases.

## *S. bovis morbificans* IN ANIMALS.

Since 1948 several times caviae and mice were found dead owing to an infection with *S. bovis morbificans* in the N.H.I. in Utrecht: once a sheep and once a fox were victims. This sheep belonged to a group of eight test animals: it died after an illness of only one day from serious enteritis. In the autopsy no striking facts could be found excepted watery contents of the intestines in which *S. bovis morbificans* could be identified. The liver and spleen gave also the same *Salmonella* in culture. The seven healthy sheep were controlled several times. In the motions of 3 *S. bovis morbificans* could be found once, but they disappeared in 10 days; the serum of one agglutinated *S. bovis morbificans* but no germs could be found in the feces. In the course of 20 days the titers with 0 antigen reached a level of 1 : 40, with specific and unspecific H-antigen one of 1 : 640. These values diminished gradually and after 4 months no agglutinins could be found.

### DETERMINATION.

The germs cultivated from the stool of the baby in question were rodlike, very motile, Gram negative with cultural and biochemical properties of the *Salmonella* group.

They fermented glucose, maltose, galactose, levulose, arabinose, mannitol, rhamnose, dulcitol, sorbitol, inositol and xylose, but lactose, saccharose, adonitol and salicin remained unchanged. No indol was formed, $H_2S$ was produced and gelatin was liquified.

The culture has been examined serologically by means of VI, VII and VI, VIII serum with positive results and the conclusion must be that VI-antigen was present.

A positive agglutination followed with VI, VIII serum saturated in a 1 : 5 dilution with VI, VII strains. It appeared that the culture was biphasic: in the beginning only a positive agglutination could be found with r-serum. The swarming method of GARD — enrichment of 1 % agar with r-serum — enabled us to develop the unspecific phase in which 1,3 and 5 antigen was present.

Following the diagnostic antigen formula of KAUFFMANN-WHITE the cultivated germ of our baby belonged to *Salmonella bovis morbificans* (VI, VIII, r-1,5). Formerly the 3 antigen was mentioned in this strain but nowadays it is omitted.

## Summary.

A 6 month old baby suffered from acute gastroenteritis with mucosanguinolent stools in which *Salmonella bovis morbificans* could be cultivated. The child lived together with an old crippled woman who had presented earlier a long lasting enteritis with mucosanguinolent stools. It was impossible to trace the way in which infection was acquired. Experience of *S. bovis morbificans* infections in animals is mentioned with a survey of literature.

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Baarn).

# EINE NEUE DISCOMYCETENGATTUNG AUS SKANDINAVIEN

von

**J. A. VON ARX**

(Eingegangen am 30. Oktober 1950).

Auf einer im Rahmen des 7. Internationalen Botanischen Kongresses in Stockholm organisierten Exkursion nach Jämtland habe ich einige Kollektionen von *Arctostaphylos alpina* gesammelt. In zweien davon fand sich auf abgestorbenen Blättern ein interessanter, inoperculater Discomycet, den ich nach der mir zur Verfügung stehenden Literatur nicht zu bestimmen vermochte und den ich auch in keiner der vorhandenen Gattungen zwanglos unterbringen konnte. Ich glaube deshalb, ihn als neue Art und Gattung beschreiben zu müssen und gebe vorerst eine ausführliche, lateinische und deutsche Diagnose.

## *CHAETONAEVIA* NOV. GEN.

Apothecia foliicola, matrice innata, vix vel paullo emergentia, primum globosa-clausa, postea discum planum nudantia, disciformia, depresse ellipsoidea vel obconica, laeticoloria. Excipulum tenuum, plectenchymaticum vel in parte media microparenchymaticum, pellucide olivacea brunneum, ad marginem setis rigidiusculis, hyalinis, longis, rectis, plerumque unicellularibus. Asci pro ratione magni, crassi-clavati, crassiuscule tunicati, 4-spori. Sporidia parallele disposita, magna, fusoidea, diu hyalina, continua, postea olivacea vel brunnea, medium septata. Paraphyses filiformes, simplices vel parum ramosae, apice sensim incrassatae, hyalinae, ascos superantes, sed epithecium typicum non formantes.

Saprophyten auf Blättern. Apothecien klein, sich im Substrat entwickelnd, kaum oder nur schwach hervorbrechend, erst kugelig geschlossen, dann sich scheibenförmig öffnend und die flache Fruchtschicht entblössend. Das Excipulum ist ziemlich dünn, überall etwa gleich dick oder in der Mitte etwas mächtiger, plektenchymatisch oder in der Mitte undeutlich kleinzellig, hell-olivbraun, an den Seiten oft etwas dunkler. Es trägt einen Ring von meist geraden, steif abstehenden, ziemlich langen, hyalinen Borsten. Die ziemlich grossen Asci sind dick-keulenförmig, derbwandig, 4-sporig. Die parallel nebeneinander liegenden Sporen sind spindelförmig, erst sind sie hyalin und einzellig, später färben sie sich mehr oder weniger dunkel und erhalten in der Mitte eine Querwand. Die fadenförmigen Paraphysen sind einfach oder im obern Drittel verästelt und oben keulenförmig angeschwollen; sie sind hyalin und überragen die Asci etwas, bilden aber kein typisches Epithecium.

## *CHAETONAEVIA NANNFELDTII* NOV. SPEC.

Apothecia hypophylla, late et dense dispersa, saepe bina complurave dense aggregata, intraepidermalia, primo globosa-clausa, mox erumpentia et discum planum pellucide fuscum nudantia, 145—190 μ diam., 80—100 μ alta. Excipulum 15—25 μ crassum, plectenchymaticum vel in parte media parenchymaticum, e cellulis indistincta, sat tenuiter tunicatis, pallide olivaceo-brunneis compositum; ad marginem setis hyalinis, crasse tunicatis, acutis, 60—90 μ longis, 3—6 μ crassis, plerumque unicellularibus obtectum. Hypothecio hyalino, fibroso, indistincto. Asci crassi clavati vel cylindracei, antice late rotundati, postice in stipitem crassum attenuati, crassiuscule tunicati, 4 spori, (50—65) × (15—22) μ. Sporidia parallele disposita, fusoidea, utrinque leniter attenuata, obtusa, inaequilatere vel plus minusve curvula, rarius recta, diu hyalina et continua, postea olivacea vel brunnea et medium septata, (43—54) × (7—10) μ. Paraphyses sat numerosae, simplices vel parum ramosae, septatae, 1—2 μ crassae, apice sensim clavato-dilatatae et rotundatae, 4 μ crassae, hyalinae, ascos superantes.

Hab. in foliis emortuis *Arctostaphyli alpinae*. Suecia, Jämtlandia, Storlien, prope frontibus Norwegii, Stenfjeldet. 24. 7. 1950. ipse legit.

Die Fruchtkörper wachsen blattunterseits unregelmässig zerstreut, stehen oft in kleineren Herden, sind aber nie miteinander verwachsen. Von blossem Auge oder auch mit der schwachen Lupe sind sie nicht erkennbar. Sie entwickeln sich intraepidermal, sind zuerst kugelig geschlossen, brechen dann hervor und öffnen sich durch unregelmässiges Aufspalten der Deckschicht mehr oder weniger rundlich-kreisförmig, so die gelbliche oder hellbraune Fruchtschicht entblössend. Sie messen 145—190 μ im Durchmesser und werden (die Borsten nicht mitgerechnet) 80—100 μ hoch.

Das Excipulum ist unten und seitlich regelmässig etwa 15—25 μ dick. Es besteht aus mehreren Lagen von unregelmässigen und undeutlichen, zusammengedrückten, dünnwandigen, hell-olivbraunen, am äusseren Rande oft etwas dunkler gefärbten Zellen und geht nach innen in das undeutlich und schwach entwickelte, von diesem nur unscharf abgegrenzte, aus hyalinen, faserigen Zellen bestehende Hypothecium über. In der Mitte ragt das Excipulum oft etwas zapfenförmig nach unten in das Substrat

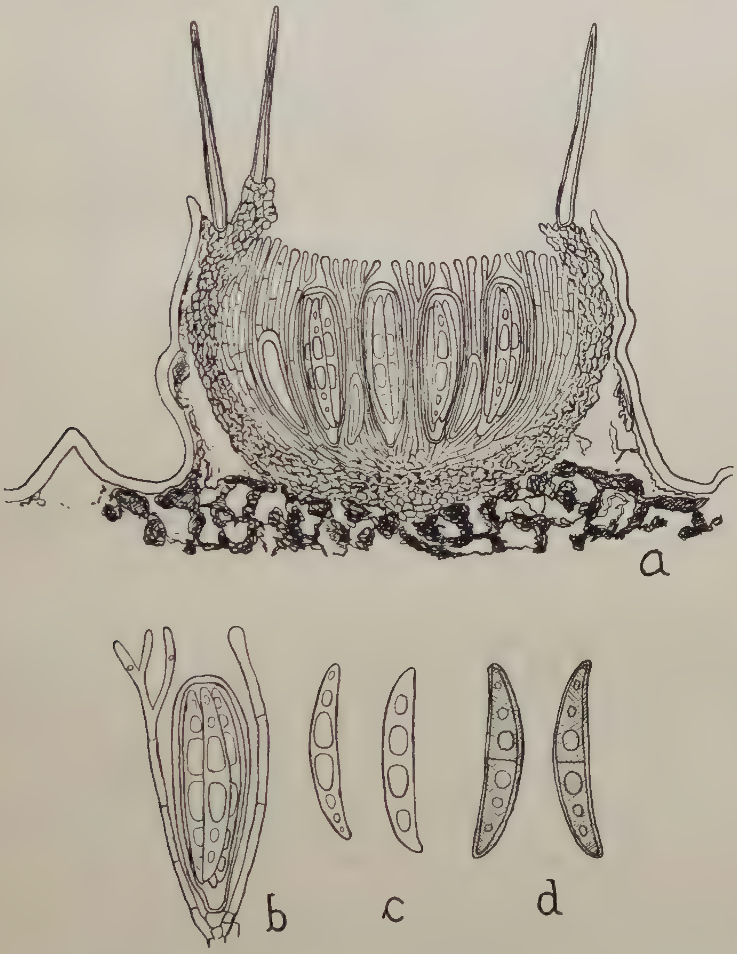

Abb 1. *Chaetonaevia Nannfeldtii* nov. spec.
*a.* Medianer Schnitt durch ein reifes Apothecium. *b.* Ascus mit Paraphysen. *c.* unreife, noch hyaline Ascosporen. *d.* reife Ascosporen. Vergr.: *a* 375 ×, *b*, *c* und *d* 500 ×.

hinein. Am äusseren Rande sitzt ihm ein Ring von Borsten auf. Diese sind ziemlich starr, mehr oder weniger senkrecht abstehend, hyalin, nicht septiert und besitzen eine zirka 1 μ dicke, stark lichtbrechende Membran. Sie sind 60—90 μ lang, unten etwa 6 μ dick, laufen nach oben bis zu einer Dicke von etwa 1 μ spitzlich zu und sind dann abgerundet.

Die Fruchtschicht ist etwa 60 μ dick. Die Asci sind breit keulenförmig oder fast tonnenförmig, oben breit abgerundet, unten in einen kurzen und dicken Stiel verschmälert; sie besitzen eine derbe und ziemlich dickwandige Membran, enthalten 4 Sporen und sind (50—65) × (15—22) μ gross. Die Sporen liegen gebündelt nebeneinander, sind spindelförmig, in der Mitte am breitesten, nach den Enden verjüngt und abgerundet, selten gerade, meist ungleichseitig oder etwas gekrümmt (dies vor allem an den Enden: *Fusarium* ähnlich) und messen (43—54) × (7—10) μ. Jung sind sie hyalin, einzellig und enthalten meist 2 grosse und mehrere kleine Oeltropfen; reif besitzen sie in der Mitte eine echte Querwand; selten kommen noch sekundäre Plasmateilungen vor; nach und nach, oft erst ausserhalb des Schlauches, bekommen sie eine olivbraune Farbe und sind zuletzt ziemlich dunkel; sie besitzen dann ein deutliches Epispor. Die ziemlich zahlreichen Paraphysen überragen die Asci etwas; sie sind hyalin, fadenförmig, mit einigen Septen, 1—2 μ dick; oft sind sie im obern Drittel etwas verzweigt, oft einfach und dann bis 4 μ keulig verbreitert und abgerundet.

*Chaetonaevia* ist eine durch ihren Bau sehr ausgezeichnete Gattung. Wie schon ihr Name sagt, gehört sie zu den *Naevioideae* (vgl. NANNFELDT (1)). Am nächsten ist sie wohl mit *Laetinaevia* Nannf. verwandt, unterscheidet sich aber von dieser Gattung durch die sich im Alter färbenden Sporen und vor allem durch den Besitz langer, hyaliner Borsten. Durch dieses Merkmal ist sie auch von den Formen verschieden, die REHM (2) in der Gattung *Phragmonaevia* zusammenfasst und die NANNFELDT (1) in verschiedene Gattungen verteilt.

Diesen schönen Pilz widme ich Herrn Prof. Dr J. A. NANNFELDT in Uppsala. Unter seiner ausgezeichneten Leitung stand die eingangs erwähnte Exkursion. *Chaetonaevia Nannfeldtii* wächst ziemlich reichlich und gut entwickelt in Gesellschaft von *Thekopsora sparsa* (Winter) Magnus und einer leider schlecht entwickelten *Gibbera* Fries.

## Summary.

*Chaetonaevia* is a new genus of the inoperculate discomycetes and is related to *Naevia* Fr. and *Laetinaevia* Nannf. The described monotypic species *Ch. Nannfeldtii* grows on dead leaves of *Arctostaphylos alpina* and was collected in Sweden.

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(From the Department of Bacteriology, N.V. Philips-Roxane, Weesp).

# THE INCIDENCE OF HAEMAGGLUTINATION INHIBITING ANTIBODIES FOR INFLUENZA A AND B VIRUS STRAINS IN SERA OF CHILDREN AND INFANTS

by

**J. VAN DER VEEN**

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Almost all influenza A outbreaks since 1947 have been associated with the new type of A virus known as A-prime. This holds true for individual influenza infections in 1947 and the more extensive 1949-epidemic of influenza in Holland (VAN DER VEEN and MULDER (6)). During the Canadian Arctic epidemic of 1949, however, influenza A virus related to the former PR8 strain (1934 U.S.A.), was isolated (VAN ROOYEN e.a. (5)). In France there have been recorded recoveries of strains of both sub-groups (A and A-prime) from the epidemic of 1949 (LÉPINE e.a. (2)). Little is known about the former incidence of influenza B infections in Holland and the serological characteristics of the causative virus strains.

Further information about the type of occurring virus strains may be obtained from an investigation of the incidence of antibodies for different influenza virus strains in sera of children and infants.

The present paper reports the results of antibody titrations of sera, collected from young children in Holland.

## Sera.

Blood samples of eighty normal children were taken in Amsterdam, during the summer of 1949 and kindly sent to us by Dr M. G. STRONK (Binnen Gasthuis, Amsterdam). Moreover, sera of thirty-seven whooping cough patients were obtained in 1950 through the courtesy of Dr J. E. MINKENHOF (Wilhelmina Gasthuis, Amsterdam) and another twenty-one sera of normal children were collected in Amsterdam during the spring of 1950. As the age of the children varied from 0 to 5 years, their sera could not contain antibodies resulting from clinical or subclinical influenza A or B infections before 1945. The children had never been vaccinated against influenza. The sera were stored without preservative at — 20° C.

In order to obtain clear results the non-specific inhibitors in the sera were eliminated by treating them with a crude filtrate of *V. cholerae* (Burnet and Stone (1), van der Veen and Mulder (6)). The capacity of the cholera filtrate to destroy the non-specific inhibitors completely in serum-dilutions of 1 to 12 was checked with normal ferret and rabbit sera.

Technique.

The following strains were used:

influenza A : PR8 (1934 U.S.A.), passage formula: $F_{198}M_{593}$ $E_{91}$ and $E_{92}$;
influenza A-prime: FM1 (1947 U.S.A.), passage formula: $EM_8E_{76}$;
influenza B : Lee (1940 U.S.A.), passage formula: $F_8M_{137}E_{139}M_{18}E_2$ and
B (1950 Ned), passage formula: $E_{11}$ [1]).

Passage of each strain was routinely made by the allantoic route in 11-day-old chick embryos. The eggs were incubated at 35° C. for 2 days. Positive allantoic fluids were pooled and used as antigens in the haemagglutination inhibition tests.

The antibody titrations were performed by a slightly modified form of the Hirst-Salk technique, *viz.* a micromethod, using tiles of porcelain with concavities (Mulder and Goslings (3); van der Veen and Mulder (6)). A series of twofold serum dilutions was made in saline and next mixed with half a volume (two drops) of virus suspension. After interaction for half an hour at 2° C. two drops of a 2 per cent chicken red cell suspension were added, the final concentration of red cells being 0.5 per cent. Readings were made after the tiles had remained undisturbed for half an hour at 2° C. The titres were expressed as the reciprocal of the highest final dilution of serum that partially inhibited 3 units of haemagglutinin.

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[1]) This strain was isolated in Holland during an outbreak of influenza B in April, 1950 and obtained through the courtesy of Prof. Mulder, Leyden. In preliminary experiments (Mulder (personal communication)), the strain proved to be antigenically identical to 7 other B strains isolated in the same period, and to a strain of B-virus recovered in 1949 in Leyden. As this group of strains deviated very much from the classical B-strain, Lee (1940 U.S.A.), the incidence of antibodies for one of them, B (1950 Ned), was investigated by us.

The titrations of all sera against one of the above mentioned strains were performed in one, two or three experiments. In each experiment the employed strain was titrated with a homologous reference antiserum, in order to check the identity of the strain and to permit comparison of the two or three tests.

## Results.

As the experiments were done with a view to investigate whether serum antibodies against each of the employed strains did occur or not, the exact level of antibodies was of less importance. Therefore we divided the results of the titrations in two groups, *viz.* sera with titres lower than 12 and higher than 12 (table 1).

Table 1.

Results of haemagglutination inhibition tests with sera collected from children under five years, against different influenza virus strains.

| | Percentage of sera containing antibodies for virus strains (titres higher than 12) | | | | Total number of investigated sera |
|---|---|---|---|---|---|
| | PR8 (1934 USA) | FM1 (1947 USA) | Lee (1940 USA) | B (1950 Ned) | |
| Sera collected in 1949 | 5% | 70% | 0% | 0% | 80 |
| Sera collected in 1950 | 2% | 63% | 0% | 0% | 43 |
| Sera collected in 1950 from infants under one year | 13% | 7% | 20% | 0% | 15 |
| Total | 5% | 61% | 2% | 0% | 138 |

Only in a few sera antibodies against the former strains PR8 and Lee were found. The sera with antibodies for Lee and two of the sera with antibodies for PR8 were obtained from infants under three months. The titres were low, varying from 12 to 96. Possibly, the antibodies were derived from the mother, which should be examined more extensively in sera of new born infants.

The other sera with antibodies for PR8, collected from older children, always contained a greater amount of antibodies for the FM1 strain. Presumably, the slight antigenic relationship between strains of the A and A-prime subgroup is the cause of the appearance of antibodies for PR8 in some sera after infection with an A-prime strain.

None of the investigated sera contained antibodies for the freshly isolated influenza strain B (1950 Ned). Localized outbreaks of influenza B have been reported in Holland during the spring of 1950 (MULDER (4)). It is probable from our experiments that in Amsterdam infections with virus related to B (1950 Ned), did not occur between 1945 and 1950 in children under five years or, if any, in a very low percentage.

Most sera contained antibodies for FM1 with titres ranging from 12 till 1536 (table 2).

Table 2.

Haemagglutination inhibiting antibodies of sera collected from children under five years, against the strain FM1 (1947 USA).

| | Titres against FM1 | | | | | | | | Total number of sera |
|---|---|---|---|---|---|---|---|---|---|
| | <12 | 12-24 | 24-48 | 48-96 | 96-192 | 192-384 | 384-768 | 768-1536 | |
| Nos of sera | 54 | 8 | 14 | 19 | 23 | 14 | 4 | 2 | 138 |

Fourteen of the fifteen infants under one year had no antibodies for FM1. Apparently, these infants, born after the 1949-epidemic of influenza A-prime, have not been exposed afterwards to A-prime virus. The serum of one infant that was younger than four months, contained, however, antibodies for FM1. Presumably they were derived from the mother. In the older age groups, the numbers of sera containing antibodies for FM1, varied from 60 to 80 per cent.

## Conclusion.

As shown by the present data, about 60 per cent of the sera of 138 children contained antibodies for influenza A-prime virus (FM1-strain). It can be safely assumed, that these antibodies result from previous exposure to influenza A-prime virus, having led to a clinical, or perhaps more frequently, to a subclinical infection.

The incidence of serum antibodies for the influenza A strain PR8, and for the influenza B strains Lee and B (1950 Ned) was very low or completely absent. The presence of antibodies for PR8 and Lee in some of the investigated sera can be possibly accounted for by a passive transfer of antibodies from the mother. In other cases it may be assumed that antibodies for PR8 have been produced after an infection with an A-prime strain as a result of the slight antigenic relationship between A-prime and A strains.

Thus, it appears that in Amsterdam there have been no recent clinical or subclinical infections with influenza virus related to PR8, Lee or B (1950 Ned). Also, it is highly improbable that in Holland strains related to the A (PR8)-subgroup have taken any part in the 1949-epidemic of influenza A-prime, which is in concordance with the fact that all 45 strains isolated in 1949 in Holland belonged clearly to the A-prime type (MULDER, personal communication).

## Summary.

The incidence of antibodies for influenza A (PR8) and influenza B virus (Lee and B (1950 Ned)) in the sera of 138 children from 0 to 5 years was very low. 60 Per cent of the investigated sera contained antibodies for influenza A-prime virus (FM1).

## Acknowledgments.

The author is grateful to Miss A. GALESLOOT and Miss A. VAN DAM for their technical assistance.

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(From the Department of Bacteriology, N.V. Philips-Roxane, Weesp).

# COMPARISON OF SOME IMMUNOLOGIC PROPERTIES OF DIFFERENT *HAEMOPHILUS PERTUSSIS* STRAINS

by

**J. VAN DER VEEN**

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The role of the immunologic properties of *H. pertussis* in the protection of man against whooping cough has not been wholly established at this time. It is known that freshly isolated, smooth phase-I strains (LESLIE and GARDNER (4)) have to be used for the production of a potent whooping cough vaccine. Among these strains, however, there exist such great variations, that further selection seems necessary.

Agglutination with phase-I serum, the ability to produce agglutinins in rabbits, and the capacity to induce a typical skin reaction on intracutaneous injection in the rabbit are accepted by some investigators as the only criteria for the suitability of a strain to be incorporated in a vaccine. Recently in the United States of America much value has been placed upon the capacity of Pertussis vaccines to induce active immunity in mice (KENDRICK e.a. (1)). KEOGH and NORTH (3) observed that the virulence of strains of *H. pertussis* after intranasal infection, and the protective potency (in experimental murine pertussis) of vaccines prepared from them, are related to their haemagglutinin content. They consider the haemagglutinin as one of the most important antigenic components. UNGAR e.a. (6) stated that the haemagglutinating property of *H. pertussis* strains is related to their virulence. However, KENDRICK e.a. (2) found no quantitative correlation between virulence and mouse protective properties. VERWEY and THIELE (7) observed that an extract of *H. pertussis* containing agglutinogen and toxin, was capable of protecting mice against intracerebral infection with virulent *H. pertussis*. The toxin, however, contributed scarcely if at all, to the protection against *H. pertussis* infection.

We have investigated some of the above mentioned properties of five *H. pertussis* strains. The strains # 11, # 18, # 19, # 20 and # 24 were isolated in 1949 in Amsterdam and in Rotterdam [1]). The experiments have been performed with smooth cultures used within 12 subcultures on BORDET-GENGOU medium containing 30 per cent sheep blood. In the comparative tests care was taken that suspensions of the various strains contained the same number of micro-organisms. The turbidity of the bacterial suspensions was determined with the aid of the opacity tubes of WELLCOME, after having compared these tubes with a standard vaccine containing 10000 million organisms per ml.

### Agglutination.

Studies were made to determine which dilution of a rabbit phase-I antiserum agglutinated the five *H. pertussis* strains.

Duplicate series of antiserum titrations were made. Serials of twofold serum-dilutions were prepared in tubes with saline and mixed with the same volume (0.5 ml) of a fresh suspension containing 10000 million organisms per ml of cultures which were incubated on BORDET-GENGOU medium for 48 hours. The tubes were allowed to stand for one hour at 37° C. and then overnight at 2°—4° C. The highest serum-dilution, which showed agglutination just visible with the naked eye, was accepted as titre. Three strains (# 11, # 20 and # 24) were agglutinated in dilutions of 1 : 25600 and two strains (# 18 and # 19) in dilutions of 1 : 6400.

### Agglutinogenic property.

Rabbits of the same body weight were injected intravenously with suspensions of *H. pertussis* in saline, containing 0.5 per cent formalin. Two rabbits were used for each of the five strains. The antisera of both rabbits injected with the same vaccine, showed the same titre against strain # 20. Cross-titrations against the other strains were performed with one of each pair of those antisera.

As shown in table 1, fairly strong variations occur among the strains investigated. It appears that the ability of a strain to produce agglutinins is not related to the degree in which this strain is agglutinated by antisera.

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[1]) The strains were kindly sent to us by Dr A. B. J. VAN DER PLAATS, Gem. Geneesk. Dienst, Amsterdam, and Dr H. REERINK, Sophia-Kinderziekenhuis, Rotterdam.

Table 1.

Agglutinin titrations of antisera against different *H. pertussis* strains.

| Antisera of vaccinated rabbits | Titres against *H. pertussis* strains 1) | | | | |
|---|---|---|---|---|---|
| Strain | # 20 | # 11 | # 24 | # 18 | # 19 |
| # 20 | **10240** | 5120 | 20480 | 20480 | 640 |
| # 11 | 10240 | **5120** | 40960 | 40960 | 1280 |
| # 24 | 5120 | 2560 | **10240** | 5120 | 40960 |
| # 18 | 640 | 1280 | 2560 | **1280** | 640 |
| # 19 | 640 | 80 | 640 | 640 | **10240** |

1) Titrations of each strain against the five antisera were always performed on one day.

## Toxic property.

The toxic properties of *H. pertussis* were studied by injecting, intracutaneously, suspensions of living germs in rabbits. Different dilutions, *viz.* 1000 million, 200 million and 20 million organisms of each strain per injection were used. The reactions were judged by the size of the area of redness, the infiltration and eventual necrosis at the site of injection. Strains # 18 and # 24 gave the strongest reactions in the different concentrations, strains # 11 and # 19 caused milder reactions, the effect of strain # 20 was intermediate.

## Virulence.

White Swiss mice were injected intracerebrally and intranasally with suspensions of *H. pertussis*, incubated for 30 hours at 37° C. and suspended in 1 per cent Casamino acids (Technical Difco). From each strain, a suitable range of dilutions was made and groups of six mice were used for each dilution. The volume of the intracerebral inoculum was 0.02 ml; the intranasal inoculum was 0.5 ml.

The death of the mice infected intranasally, occurred with signs of pneumonia. The mice appeared ill and ate less than normally; their breathing was laboured, and they tended to huddle together. The lungs of the mice which died after intranasal infection, showed patchy — sometimes confluent — areas of consolidation, which were reddish-brown in colour. The death of the mice, injected intracerebrally, occurred with typical signs of encephalitis. The mice lost their muscular control, became prostrated and showed convulsions. No histological or bacteriological examination of the lungs and of the brains was carried out. The results of the tests are listed as in table 2.

Table 2.

Results of virulence tests of different *H. pertussis* strains in mice.

| Strain | Number of bacteria that kills 100% of the mice | |
|---|---|---|
| | Intracerebral infection | Intranasal infection |
| # 20 | $< 8 \times 10^2$ | $< 2 \times 10^3$ |
| # 18 | $8 \times 10^5$ | $2 \times 10^4$ |
| # 24 | $8 \times 10^7$ | $2 \times 10^6$ |
| # 11 | $8 \times 10^5$ | $> 2 \times 10^8$ |
| # 19 | $> 8 \times 10^7$ | $> 2 \times 10^8$ |

For unknown reasons in successive tests, however, strains # 20 and # 18 were found to be less virulent. Larger doses, *viz.*, $8 \times 10^7$ organisms after intracerebral injection and $2 \times 10^8$ after intranasal infection were required to kill 100 per cent of the mice.

### Mouse protection.

The immunizing potency of vaccines of the five *H. pertussis* strains were investigated according to the method of KENDRICK (1). A 48 hours growth of the bacteria on BORDET-GENGOU medium was scraped from the surface of the medium, suspended in saline, centrifuged and inactivated with sodium ethylmercuri thiosalicylate. Groups of 18 mice were injected twice intraperitoneally with three different dilutions of each vaccine and after a rest period of ten days they were infected intracerebrally with a challenge culture [1]). The number of fatal results in each group was recorded and the vaccine dose capable of protecting 50 per cent of the mice (ImD/50), was calculated according to the method of REED and MUENCH (5). For comparison we have tested also a reference antigen (M 213) [1]). The dosage of the vaccines was based on photometer readings and checked with the reference antigen.

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[1]) For the challenge culture 18–323 and reference antigen M 213 we are very grateful to Dr P. L. KENDRICK.

All the experiments were made at the same time on white Swiss mice, 14–16 g in weight. Control groups of mice were injected intraperitoneally with saline. The mice were infected intracerebrally with 200000 bacteria of the challenge culture. Moreover, in order to establish the LD/50, mice of the control group were injected with stronger dilutions of the challenge culture.

The numbers of organisms of the various vaccines required to protect 50 per cent of the mice, were listed as in table 3. The ratios of the ImD/50 values of the tested vaccines to the ImD/50 value of the reference vaccine were calculated and also listed in the table.

Table 3.

Results of mouse protection tests with different *H. pertussis* vaccines.

| Vaccine Strain | ImD/50 values | Ratio: tested vaccine to reference vaccine |
|---|---|---|
| # 20 | 880 million | 1.1 |
| # 11 | 1040 million | 1.3 |
| # 24 | 1440 million | 1.8 |
| # 19 | 1440 million | 1.8 |
| # 18 | 2000 million | 2.5 |
| reference vaccine | 800 million | = 1 |

LD/50 of challenge culture 18–323, used in these tests, is 640 organisms.

It is obvious that the tested vaccines differed in immunizing potency. As we have not determined what variations in protective properties of one vaccine may occur from test to test, the significance of the observed differences cannot be estimated.

## Haemagglutinin.

The haemagglutinating properties of *H. pertussis* were studied according to the method of Keogh and North (3). Fresh suspensions of strains (10000 million organisms per ml) were diluted in tubes with saline containing 0.01 per cent cysteine hydrochloride, neutralized with sodium hydroxyde and mixed with the same amount of washed fowl erythrocytes up to a final concentration of 0.25 per cent red cells. After the tubes had been standing overnight at room temperature, the patterns at the bottom were read. The haemagglutinin titres of the five *H. pertussis* strains lay between 0 and 8. Using red cells of guinea-pigs instead of fowl erythrocytes, somewhat higher titres, *viz.* 16 or 32, were found. Also in tests of 20 other freshly isolated *H. pertussis* strains we have noted low titres varying within a very small range.

### Antibody response of vaccinated children[1].

It was not possible for us to compare the antigenicity of the vaccines of each of the five strains in children. Only one vaccine, *viz.* of strain # 20, could be investigated. The ImD/50 of this vaccine was 0.36 × ImD/50 of the reference antigen.

33 Children, from 2 – 5 years, who had not had whooping cough, were vaccinated by three injections with intervals of four weeks. The total dose of organisms administered, ranged from 60000 to 90000 million. Blood was taken four weeks after the last injection. The agglutination titre of the obtained sera was determined with a freshly isolated phase-I *H. pertussis* strain, different from the strain contained in the vaccine. Table 4 shows the results of the titrations.

Table 4.

Results of agglutinin titrations of 33 sera, obtained from children vaccinated with *H. pertussis* vaccine of strain # 20.

| | Agglutinin titres | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | <20 | 20 | 40 | 80 | 160 | 320 | 640 | 1280 | 2560 |
| Nos of sera | 0 | 0 | 1 | 2 | 2 | 11 | 8 | 6 | 3 |

### Conclusion.

The experiments made show that fairly strong variations occur in the properties of five *H. pertussis* strains investigated. It is not possible to demonstrate a clear correlation between some of these characteristics. However, it seems that the ability of strains to produce phase-I agglutinins in the rabbit is related to their protective potency in experimental murine pertussis. Presumably both these properties have been the accepted criteria for selecting strains for the preparation of a vaccine.

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[1] These experiments have been performed in a nursing home at Amsterdam. We are indebted to Dr PH. H. FIDELDY DOP and Dr G. RÜBSAAM, Emma Kinderziekenhuis, Amsterdam, for their co-operation.

In our experiments one strain was found to be superior to the others. This strain was chosen for the vaccine production. The antibody-response of children, injected with this vaccine, was satisfactory. Four weeks after the last injection 28 of 33 vaccinated children showed titres higher than 300.

## Acknowledgments.

The author is very much indebted to Prof. Dr A. Ch. Ruys, Amsterdam, for her interest and advice. He is grateful to Miss A. van Dam for her technical assistance.

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# ACQUISITION DE PROPRIETES ANTIBIOTIQUES NOUVELLES PAR LA SOUCHE *E. COLI* V SOUS L'ACTION DES BACTERIOPHAGES T.1, T.5 ET T.7

par

**P. FREDERICQ** [1]

(Reçu le 7 décembre 1950).

En étudiant la sensibilité à divers bactériophages de souches antibiotiques d'Entérobactériacées, nous avons constaté que leurs mutants résistant aux bactériophages conservent en général une action antibiotique absolument identique à celle de la souche-mère dont ils dérivent. Dans le cas de la souche *E.coli* V de GRATIA (3) cependant, nous avons constaté que, si les mutants résistant aux phages T.1, T.5 ou T.7 de DEMEREC et FANO (1) continuent certes à produire la colicine V caractéristique de la souche V dont ils dérivent, ils ont de plus acquis la propriété nouvelle de produire simultanément une deuxième colicine.

L'expérience est réalisée de la manière suivante: Nous ensemençons sur deux boîtes d'eau peptone gélosée, par piqûre au centre de chacun des quadrants, la souche V et les mutants V/1, V/5 et V/7 (mutants de V résistant aux phages T.1, T.5 ou T.7). Après 48 heures d'incubation à 37°C., nous stérilisons ces cultures par des vapeurs de chloroforme puis nous ensemençons toute la surface à l'aide d'un disque de papier filtre imbibé d'une culture en bouillon de la souche indicatrice sensible *E.coli* $\varphi$ sur une boîte et d'un mutant de $\varphi$ résistant à la colicine V (désigné $\varphi$/V) sur l'autre boîte. Après nouvelle incubation de 24 heures, on constate que, sur la boîte ensemencée de $\varphi$, la souche V et les mutants V/1, V/5 et V/7 déterminent une large zone d'inhibition de 38 à 40 mm de diamètre, caractéristique de la colicine V. Sur la boîte ensemencée de $\varphi$/V, la souche V ne détermine plus de zone d'inhibition, ce qui est normal puisque la souche V ne produit que la colicine V à laquelle le mutant φ/V n'est plus sensible, mais les mutants V/1, V/5 et V/7 présentent encore une zone d'inhibition de 15 mm de diamètre, trahissant ainsi la production par ces mutants d'une deuxième colicine dont l'action sur φ était masquée par la zone de diffusion beaucoup plus large de la colicine V.

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[1] Associé du Fonds National de la Recherche Scientifique.

Nous avons recommencé cette expérience en faisant agir ces souches non plus sur φ ou sur φ/V, mais sur des mutants de φ/V repiqués des zones d'inhibition produites par V/1, V/5 ouV/7. Ces mutants de φ/V, sélectionnés par V/1, V/5 ou V/7, sont tous résistant à la fois à l'action de V/1, V/5 et V/7, ce qui prouve donc que la nouvelle colicine est identique dans les trois cas; les zones d'inhibition produites sur φ/V par V/1, V/5 ou V/7 ont d'ailleurs une morphologie tout à fait semblable. Cette nouvelle colicine produite par les mutants de V résistant aux phages T.1, T.5 ou T.7 sera, pour la facilité de l'exposé, désignée colicine M.

La colicine M est distincte de la colicine V. Nous venons en effet de voir qu'elle est beaucoup moins diffusible. Elle a de plus une spécificité très différente puisque son action est révélée par les mutants de type φ/V totalement résistant à la colicine V. Son spectre d'activité est également très différent comme le montre le tableau suivant:

| | Inhibition produite par les souches: | |
|---|---|---|
| | V | V/1, V/5 ou V/7 |
| Sur *E.coli* φ | Type V | Type V |
| Sur id.résistant à V | — | Type M |
| Sur *E.coli* B | Type V | Type V |
| Sur id.résistant à V | — | — |
| Sur *E.coli* C.18 | — | Type M |
| Sur *E.coli* YR | — | — |

On voit donc d'après ce tableau que la colicine M produite par V/1, V/5 ou V/7 inhibe la souche C.18 qui n'est pas sensible à la colicine V ainsi que le mutant de φ résistant à la colicine V, mais est sans action sur la souche YR et le mutant de B résistant à la colicine V; les souches-mères φ et B, sensibles à la colicine V, sont évidemment inhibées par V/1, V/5 et V/7 par l'intermédiaire de leur colicine V plus diffusible. L'action de V/1, V/5 et V/7 a été étudiée sur 19 autres souches comprenant les espèces *E.coli*, *Sh. sonnei*, *S. schottmuelleri* et *S.gallinarum* avec des résultats analogues. *S.schottmuelleri* par exemple qui n'est que peu ou pas sensible à la colicine V est inhibé par la colicine M, tandis que *Sh.sonnei*, très sensible à la colicine V, n'est pas inhibé par la colicine M. La colicine M est donc distincte de la colicine V; elle est aussi distincte des 16 autres colicines précédemment identifiées (2). En effet, si nous étudions la sensibilité aux 17 colicines de mutants du type $\varphi/VM$ (obtenus par résistance de $\varphi/V$ à la colicine M), nous constatons qu'ils se comportent vis-à-vis de ces 17 colicines exactement comme la souche $\varphi/V$ dont ils dérivent et que, par conséquent, la résistance à la colicine M est indépendante de la résistance aux autres colicines déjà identifiées.

La propriété de produire la colicine M que manifestent les mutants de coli V résistant aux phages T.1, T.5 ou T.7 est un caractère nouveau, stable et héréditaire; elle traduit donc une mutation. Cette mutation est liée spécifiquement à l'action des phages T.1, T.5 ou T.7 à l'exclusion de tout autre et peut être reproduite à volonté. Nous avons en effet étudié 70 mutants de coli V résistant à divers bactériophages; les 50 mutants sélectionnés par les phages T.1, T.5 ou T.7 produisent tous la colicine M en plus de la colicine V, tandis que les 20 mutants sélectionnés par d'autres bactériophages ne produisent aucun la colicine M.

Les 3 phages T.1, T.5 et T.7 diffèrent par leurs propriétés morphologiques et sérologiques et par leur comportement vis-a-vis de leur souche hôte *E.coli* B, mais ils exercent tous trois une activité lytique identique sur la souche *E.coli* V: les taches de lyse ont la même morphologie et les colonies résistantes qui y apparaissent sont à la fois résistantes aux 3 phages. Il n'est donc pas étonnant qu'ils déterminent tous trois l'apparition d'un mutant antibiotique du même type.

Les phages T.1, T.5 ou T.7 déterminent l'apparition du mutant de V produisant la colicine M, mais, une fois que ce mutant est apparu, les phages ne jouent plus aucun rôle. Nous avons recherché si les cultures des mutants V/1, V/5 ou V/7 continuent à reproduire du bactériophage, c'est-à-dire sont lysogènes. Dans la plupart des cas, il n'est pas possible de trouver du bactériophage dans les cultures de ces mutants et, dans les rares cas où l'on en trouve, il est très facile de les en débarrasser par isolement sur gélose, sans modifier leur propriété antibiotique nouvelle.

L'étude d'un mutant V/5 lysogène nous a permis de faire une constatation très intéressante. Lorsqu'on étudie son action sur $\varphi$ ou sur $\varphi/V$, on voit souvent apparaître, en dehors des zones d'inhibition produites par les colicines V ou M respectivement, des taches isolées de lyse. Ces taches isolées résultent de la dissémination de particules de phage à partir de la culture de V/5 au moment où on applique le papier filtre portant les souches indicatrices $\varphi$ ou $\varphi$/V. Or nous avons constaté que ces taches produites par le phage T.5 que libère la culture V/5 n'apparaissent jamais lorsqu'on fait agir V/5 sur $\varphi$/VM, c'est-à-dire sur un mutant issu de $\varphi$/V et sélectionné par l'action de la colicine M. De fait, le mutant $\varphi$/VM s'est révélé totalement résistant aux phages T.1 et T.5 et partiellement au phage T.7, alors que $\varphi$ et $\varphi$/V sont sensibles à ces 3 phages. Nous avons constaté pour d'autres mutants, issus de $\varphi$/V ou d'autres souches, que les mutants sélectionnés par la colicine M sont également résistant aux phages T.1 et T.5 et que, inversement, les mutants sélectionnés par T.1 ou T.5 sont également résistant à la colicine M. A vrai dire, cette résistance croisée n'est pas toujours absolue car si tous les mutants sélectionnés par T.1 sont totalement résistant à la colicine M, certains des mutants sélectionnés par la colicine M conservent une sensibilité tout au moins partielle aux phages T.1 et T.5; il y a donc résistance croisée mais avec des différences qualitatives dans le degré de résistance que manifestent ces mutants.

La résistance croisée entre phage T.1 et colicine M permet de vérifier l'indépendance de la sensibilité aux colicines V et M chez la souche *E.coli* $\varphi$. En effet, la résistance totale de la souche $\varphi$ à l'action antibiotique du mutant V/1 qui produit les colicines V et M, peut être obtenue indifféremment en sélectionnant par la colicine M ou le phage T.1 un premier mutant déjà résistant à la colicine V, ou bien en sélectionnant par la colicine V un premier mutant déjà résistant au phage T.1 et, par conséquent, à la colicine M. Nous résumons dans le tableau suivant les différents types d'inhibition produits par le coli V, son mutant V/1 et le phage T.1 sur la souche coli $\varphi$ et différents mutants qui en dérivent:

Inhibition produite par:

| | V | V/1 | T.1 |
|---|---|---|---|
| Sur $\varphi$ | type V | type V | lyse ++ |
| „ $\varphi$/1 | type V | type V | lyse — |
| „ $\varphi$/V | — | type M | lyse ++ |
| „ $\varphi$/V,M | — | — | lyse — ou + |
| „ $\varphi$/1,V | — | — | lyse — |
| „ $\varphi$/V,1 | — | — | lyse — |

En conclusion, on voit donc apparaître sous l'action des phages T.1, T.5 ou T.7 sur la souche *E. coli* V un mutant produisant, outre la colicine V caractéristique de la souche-mère dont il dérive, une deuxième colicine entièrement distincte. Cette nouvelle colicine, désignée colicine M, agit sur certaines souches sensibles par l'intermédiaire de récepteurs communs aux phages qui ont déterminé sa production.

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(From the Laboratory of Hygiene of the University, Utrecht).

# ON THE ACTION OF SULFANILAMIDE

## XIV. Growth of a para-aminobenzoic acid requiring *E. coli* without para-aminobenzoic acid [1]

by

**P. G. DE HAAN and K. C. WINKLER**

(Received December 14, 1950).

In a previous paper (Winkler and de Haan (9)) it was shown that the action of high concentrations of sulfanilamide against *E. coli* can be antagonized non competitively by the addition of four non competitive antagonists: methionine, xanthine, serine and thymine (or folic acid). In further experiments (de Haan, Winkler and Baljet (3)) valine was shown to be the fifth antagonist in this series. The addition of each antagonist gave a stepwise decrease of the para-aminobenzoic acid concentration which was necessary to antagonize the sulfanilamide (increase of the antibacterial index), later members of the series being inactive, unless the former were present. Again the addition of each antagonist to a series of cultures with varying concentrations of sulfanilamide gave a stepwise increase of the sulfanilamide tolerance (Winkler, de Haan and van de Langerijt (10)).

According to the theory of inhibition analysis these results were taken as evidence that the five substances were the endproducts of five para-aminobenzoic acid-dependent reactions which are inhibited by sulfanilamide. The sulfanilamide sensitivity decreases from the methionine-synthesizing system to the thymine or valine-producing chain of reactions. On further evidence (de Haan (2)) it is supposed that some intermediate is formed from para-aminobenzoic acid, which acts as part of the enzyme(s) synthesizing the final product (see diagram on p. 117).

One should expect that the five non competitive antagonists which replace para-aminobenzoic acid as a sulfanilamide antagonist will also be able to replace para-aminobenzoic acid as a growth factor in a para-aminobenzoic acid requiring mutant. Experiments with such a strain are reported in this paper. LAMPEN, ROEPKE and JONES (5) studied an analogous case whereas CUTTS and RAINBOW (1) reported related results with a para-aminobenzoic acid-less yeast.

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[1] XIII: Antonie van Leeuwenhoek **15**, 136, 1949.

## EXPERIMENTAL.

Two strains of *E. coli* were used throughout these investigations: *E. coli* 273, a para-aminobenzoic acid-less mutant strain obtained by irradiation with X-rays and the parent strain *E. coli* 518. The mutant strain was isolated by LAMPEN, ROEPKE and JONES (5). Both strains were obtained through the courtesy of Dr WOODS (Cambridge). The bacteria were transferred weekly on broth-agar. A standard droplet (0.03 ml) of a 1 : 100 dilution of a 24 hour-culture of *E. coli* on synthetic medium (in the case of *E. coli* 273 with 10 $\mu$ g/l para-aminobenzoic acid) was used as inoculum for 10 ml of the final medium. The medium (double strength) contained: glucose 20 g; $KH_2PO_4$ 6 g; $K_2HPO_4$ 6 g; $NH_4Cl$ 2 g; $MgSO_4 . 7 H_2O$ 50 mg; $FeSO_4 . 7 H_2O$ 5 mg; destilled water 500 ml.

In order to remove traces of para-aminobenzoic acid, which were still present, the complete medium was heated during ten minutes with 25 g/l norit; after filtration the container was closed with an inverted glass beaker instead of cottonwool. Five ml of the doubly concentrated medium were pipetted in glass tubes (diameter 2.9 cm, closed with metal caps). Para-aminobenzoic acid, sulfanilamide and the non competitive sulfanilamide antagonists were added to a final volume of 10 ml. After inoculation the mixtures were incubated at 37° C. Growth was measured as turbidity in a Moll extinctometer.

## RESULTS.

### I. THE SERIES OF NON COMPETITIVE SULFANILAMIDE ANTAGONISTS FOR A PARA-AMINOBENZOIC ACID REQUIRING *E.coli* (STRAIN 273).

*E. coli* 273 was cultured in 10 ml tubes with 2000 mg/l sulfanilamide, various concentrations of para-aminobenzoic acid and constant concentrations of the non competitive antagonists as described in previous experiments. Growth was measured at 70 hours; results are plotted in fig. 1.

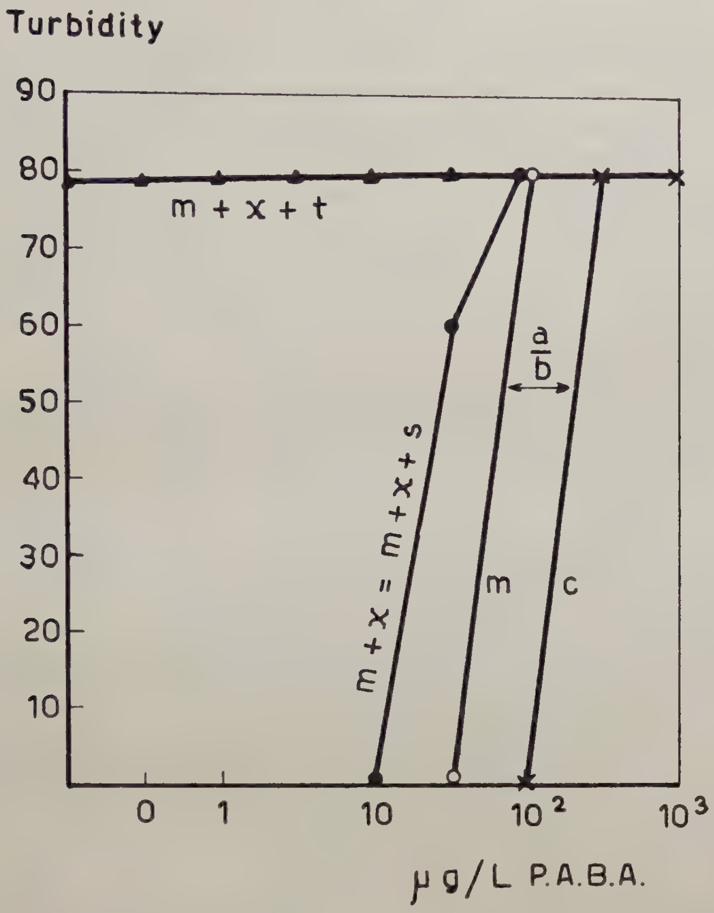

Fig. 1. Non competitive antagonists for *E. coli* 273.

Growth in synthetic medium with 2000 mg/l sulfanilamide and varying concentrations of para-aminobenzoic acid after 70 h incubation. *c* = control; *m* = methionine; *x* = xanthine; *s* = serine; *t* = thymine.

*a.* As is seen in fig. 1, a concentration of 300 μ g/l para-aminobenzoic acid is necessary to restore growth in the basal medium, whereas 100 μ g/l para-aminobenzoic acid sufficed in the presence of methionine. The increase in the antibacterial index indicates that methionine is active as the first non competitive sulfanilamide antagonist.

*b.* A further increase in the antibacterial index could be obtained by the addition of xanthine; about 30 μ g/l para-aminobenzoic acid suffices to restore growth in a medium with methionine and xanthine: xanthine acts as the second antagonist.

*c.* As is reported in a previous publication (WINKLER and DE HAAN (9)), serine is the next non competitive antagonist for some strains of *E. coli* whereas with this amino acid no shift in the antibacterial index could be obtained with *Staphylococcus aureus* (DE HAAN, WINKLER and BALJET (3)).

As is seen in figure 1 no further shift in the antibacterial index could be obtained with serine. This seems to show that serine is not active as a sulfanilamide antagonist in this case.

*d.* A further increase in the antibacterial index could however be obtained by the addition of thymine: thymine is the third non competitive antagonist.

In our previous paper, we showed that thymine could be replaced by high concentrations of folic acid (300 $\mu$ g/l). In this case however folic acid, pteroic acid and rhizopterine are inactive as sulfanilamide antagonists in a concentration of 1000 $\mu$ g/l.

*e.* As growth in the presence of 2000 mg/l sulfanilamide is restored by the 3 antagonists, it seems that the set of antagonists for *E.coli* 273 is complete. Full growth is however only obtained in about 70 hours. Normal growth in the absence of para-aminobenzoic acid was obtained in about 30 hours, when valine was added to the medium, indicating that valine was the endproduct of the fourth enzyme-system, inhibited by sulfanilamide.

## II. GROWTH OF A PARA-AMINOBENZOIC ACID REQUIRING *E.coli* WITHOUT PARA-AMINOBENZOIC ACID.

As *E. coli* 273 is able to grow in the presence of 2000 mg/l sulfanilamide without para-aminobenzoic acid, one might expect that the non competitive antagonists might replace para-aminobenzoic acid not only as a sulfanilamide-antagonist but also as a growth factor. *E.coli* 273 was incubated with various concentrations of para-aminobenzoic acid and constant concentrations of the four sulfanilamide antagonists, but without sulfanilamide.

As is seen in fig. 2, about 3 $\mu$ g/l para-aminobenzoic acid is necessary to obtain full growth of the mutant strain, whereas in the presence of methionine only 1 $\mu$ g/l para-aminobenzoic acid suffices.

A further decrease in the para-aminobenzoic acid concentration, necessary for full growth is obtained by the addition of xanthine and thymine. Indeed in the absence of para-aminobenzoic acid normal growth is obtained in about 40 hours by the addition of methionine, xanthine and thymine. These substances seem to replace para-aminobenzoic acid completely and the amino acids serine and valine are not necessary in this case. Again pteroylglutamic acid, pteroic acid and rhizopterine could not replace any of the three compounds necessary to replace para-aminobenzoic acid as a growth factor.

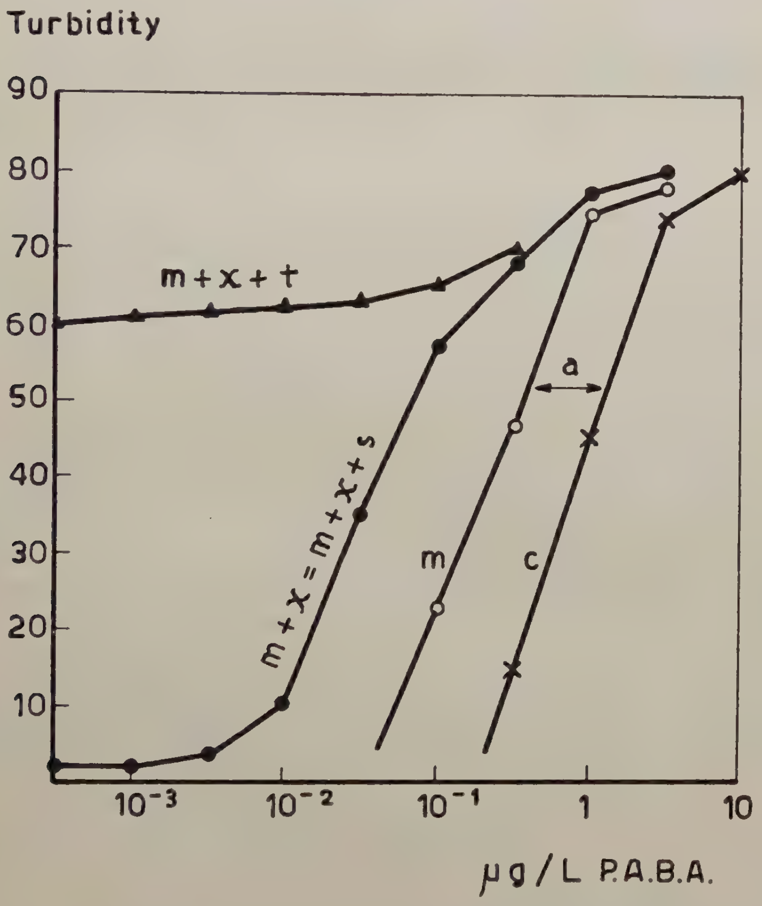

Fig. 2. The amount of para-aminobenzoic acid necessary for growth decreases stepwise on the addition of methionine (*m*), xanthine (*x*) and thymine (*t*). *E. coli*, synthetic medium, without sulfanilamide, after 40 hours incubation.

### III. NON COMPETITIVE SULFANILAMIDE ANTAGONISTS FOR THE PARENT STRAIN (*E. coli* 518).

In the presence of 2000 mg/l sulfanilamide, full growth of *E. coli* 273 is obtained with the antagonists methionine, xanthine, thymine and valine.

To establish, whether the same non competitive antagonists were active for the parent strain of *E.coli* 273, the first experiment was repeated with *E.coli* 518.

The same series of non competitive sulfanilamide antagonists was found again i.c. methionine, xanthine, thymine and valine. As in the case of *Staphylococcus aureus* serine is not a sulfanilamide antagonist for *E.coli* 273 and 518.

## DISCUSSION.

The growth requirements of the para-aminobenzoic acid-less strain was studied extensively by LAMPEN, ROEPKE and JONES (5). It was not so much our aim to study the growth requirements of the strain 273 as to draw attention to some quantitative aspects of our experiments, which are of importance for the conception of the function of para-aminobenzoic acid in the cell. The para-aminobenzoic acid requirement of *E.coli* 273 decreases stepwise on the addition of methionine, xanthine and thymine. This means that the xanthine synthesizing system is able to function in a smaller para-aminobenzoic acid-concentration than the methionine producing chain or: the dissociation constant of the para-aminobenzoic acid-enzyme complex in methionine synthesis is larger than the corresponding dissociation constants in the other two systems.

This conclusion affects the theory of inhibition analysis as given by SHIVE and MACOW (8), who state that the competitive deblocking of an enzyme by para-aminobenzoic acid depends on the equation:

$$\frac{[\mathrm{I}]}{[\mathrm{S}]} = \frac{\mathrm{K}_I}{\mathrm{K}_S} \times \frac{\mathrm{C}_{EI}}{\mathrm{C}_{ES}} = \mathrm{K}$$

I = concentration of inhibitor
S = concentration of substrate
$\mathrm{K}_I$, $\mathrm{K}_S$ are dissociation constants
$\mathrm{C}_{ES}$ = concentration of the enzyme-substrate complex ⎫ for a fixed
$\mathrm{C}_{EI}$ = concentration of the enzyme-inhibitor complex ⎭ ratio I/S

For each enzyme-system K is different, which explains the differences in sulfanilamide sensitivity. SHIVE leaves the question open however, whether the variation of K is due to a variation of $K_I$ or $K_S$ or both. A more quantitative derivation starting from the MICHAELIS-MENTEN equation (6) seems preferable.

Suppose an enzyme E synthesizing the product P by way of the enzyme-substrate-complex ES

$$E + S \rightleftarrows ES \rightleftarrows E + P \tag{1}$$

Calling the concentration of the substrate S, the total enzyme-concentration $e$, the concentration of the enzyme-substrate-complex $p$ and thus the concentration of the free enzyme $(e - p)$ we have:

$$K_S = \frac{(e—p)\,S}{p} \quad \text{and} \quad p = \frac{e.S}{K_S + S} \tag{2}$$

Supposing the reaction-velocity (V) to be dependent on the concentration of the enzyme-substrate complex $p$, we get V=cp and

$$V = \frac{ceS}{K_S + S} = \frac{ce}{\frac{K_S}{S} + 1} \tag{3}$$

V is maximal ($V_m$) at large values of S, in which case $V_m$ = ce and thus

$$V = \frac{V_m}{\frac{K_S}{S} + 1} \tag{4}$$

This is the original form of the equation of MICHAELIS and MENTEN (6); $K_S$ is the Michaelis constant. For $V = \frac{1}{2} V_m$, $K_S$ equals the numerical value of S.
At low concentrations of substrate, V will be small or $V = \alpha V_m$ $(\alpha < 1)$. Formula (4) can thus be transformed:

$$\alpha = \frac{S}{K_S + S} \tag{5}$$

Thus equation (6) will be equally valid for the methionine and the xanthine synthesizing systems:

$$\alpha = \frac{S_m}{K_m + S_m} = \frac{S_x}{K_x + S_x} \tag{6}$$

The indices $m$ and $x$ refer to the methionine or xanthine synthesizing systems. Formula (6) is only valid of course when $S_m$ and $S_x$ denote the substrate concentration at which both reaction velocities are $\alpha\, V_m$.

Equation (6) can be transformed to

$$\frac{K_m}{K_x} = \frac{S_m}{S_x} \tag{7}$$

or in words:

The dissociation constants of two enzymes vary proportionally with the substrate concentrations at which the reaction velocities (as a fractionof maximal veloctiy) are equal. This is only true of course if the amount of both enzymes is of the same order of magnitude and both are working in normal growth at about the same level of their maximal capacity.

According to (7) the ratio $\frac{K_m}{K_x}$ could be derived from fig. 1 by dividing the substrate concentration (para-aminobenzoic acid) at f.i. half maximal growth in the basal medium (curve *c*, dependent on methionine synthesis) by that in the methionine containing medium (curve *m*, dependent on the action of the xanthine synthesizing enzyme).

In fact two suppositions are introduced here : 1) the velocity of growth is dependent on the reaction velocity of the limiting enzyme and 2) the growth rates in the cultures which showed half-maximal growth were equal. It is usual to accept the first supposition. That the second one is approximately true was proved by experiment. We thus consider that the distance *a* in fig. 1 is a measure for the ratio between $K_m$ and $K_x$ and that actually $K_m > K_x$.

On the addition of the inhibitor I two reactions are involved

$$E + S \rightleftarrows ES \rightleftarrows E + P \text{ and } E + I \rightleftarrows EI \tag{8}$$

Calling the concentration of the inhibitor I, the concentration of the enzyme-inhibitor complex $q$ and writing $K_S$ and $K_I$ for the dissociation constants of the enzyme-substrate complex and the enzyme-inhibitor complex respectivily we obtain by use of the mass-law:

$$S(e-p-q) = pK_S \text{ and } I(e-p-q) = qK_I \tag{9}$$

or by elimination of *q*:

$$p = \frac{e}{\frac{I}{S} \times \frac{K_S}{K_I} + 1 + \frac{K_S}{S}} \tag{10}$$

The reaction velocity in the presence of I ($V_I$) will again be proportional to the concentration of the enzyme-substrate complex ($V_I = cp$) or

$$\frac{1}{V_I} = \frac{1}{ce}\left(\frac{I}{S} \times \frac{K_S}{K_I}\right) + \frac{1}{ce}\left(1 + \frac{K_S}{S}\right) \tag{11}$$

According to (3) the second term on the right is the reciproke of the reaction velocity in the presence of the substrate concentration S. In the presence of the high substrate concentrations used in inhibition analysis $V_S$ (the velocity in the absence of I) approaches $V_m$. As $V_m = ce$, equation (11) can be transformed in

$$\frac{V_m}{V_I} = \frac{I}{S} \times \frac{K_S}{K_I} + 1 \tag{12}$$

Supposing that for a certain ratio I/S the reaction velocity $V_I = \beta\, V_m$ ($\beta < 1$) we obtain:

$$\frac{1}{\beta} = \frac{I}{S} \times \frac{K_S}{K_I} + 1 \tag{13}$$

As this equation is equally valid for the methionine-synthesizing and the xanthine-synthesizing system (see restrictions on p. 114) we have

$$\frac{1}{\beta} = \left(\frac{I}{S} \times \frac{K_S}{K_I}\right)_m + 1 = \left(\frac{I}{S} \times \frac{K_S}{K_I}\right)_x + 1 \tag{14}$$

in which the indices *m* and *x* denote the special values of I/S at which each enzyme works at the procentual reaction velocity *β*. For 100 % inhibition I/S is the antibacterial index (*Q*) and

$$\frac{Q_x}{Q_m} = \left(\frac{K_S}{K_I}\right)_m \times \left(\frac{K_I}{K_S}\right)_x = \frac{(K_S)_m}{(K_S)_x} \times \frac{(K_I)_m}{(K_I)_x} \tag{15}$$

or in words: The increase of the antibacterial index of an inhibitor which is obtained by the addition of the endproduct of the first (most sensitive) reaction is due to the variation of the ratio of the dissociation constants of the substrate- and inhibitor-complexes of both enzymes.

For simplicity we call

$$\frac{(K_S)_m}{(K_S)_x} = a \qquad \frac{(K_I)_m}{(K_I)_x} = b \qquad \frac{Q_x}{Q_m} = \frac{a}{b}$$

Three theoretical possibilities then arise:

1. $a = b$ or $Q_x = Q_m$. Both enzymes are equally sensitive to the inhibitor and no increase of the antibacterial index will be obtained by the addition of one endproduct.
2. $a < b$ or $Q_x < Q_m$. The xanthine synthesizing enzyme would be the most sensitive one.
3. $a > b$ or $Q_x > Q_m$, which is evidently what happens in our case. The methionine-synthesizing enzyme is the most sensitive enzyme. The distance between the curves *c* and *m* in fig. 2 is indeed a measure for $\frac{Q_x}{Q_m} = \frac{a}{b}$.

We know already from fig. 1 that in our case $a = \frac{(K_S)_m}{(K_S)_x} > 1$.

For the case $a > b$ we have thus again three possibilities: $b < 1$, $b = 1$, and $b > 1$ or $a/b > a$, $a/b = a$ and $a/b < a$.

Comparing figures 1 and 2 it is evident that the distance between the curves *c* and *m* (and eventually between *m* and *x*) are of the same order. Thus $a/b$ is indeed equal to $a$, which means that $b$ is not far from unity. This proves that the dissociation-constants $(K_I)_m$ and $(K_I)_x$ are approximately equal.

We may then conclude that the observed difference in sensitivity between the reaction chains leading to methionine, xanthine and thymine is not a difference in sensitivity for sulfanilamide (as $(K_I)_m = (K_I)_x$) but a difference in sensitivity for para-aminobenzoic acid as $(K_S)_m > (K_S)_x > (K_S)_t$.

Less formally put, we observed a stepwise decrease of the para-aminobenzoic acid requirement of *E.coli* 273 by the addition of methionine, xanthine and thymine, which means that each of the three reaction chains works at a different para-aminobenzoic acid concentration (Exp. II). On the addition of sulfanilamide (Exp. I) only a shift in the necessary para-aminobenzoic acid concentration occurs, but the ratio between the para-aminobenzoic acid concentrations at which each enzyme system works is unchanged (The distances between the curves in fig. 1 and fig. 2 are approximately equal). Thus there is no differential sensitivity with regard to sulfanilamide between the reaction chains involved but only the originally existing difference in para-aminobenzoic acid sensitivity.

This conclusion has far reaching consequences. According to the original suppositions of SHIVE several reactions, starting from the same substrate were inhibited by the inhibitor. In our case it seems rather odd that three enzymes should combine with one substrate with very different avidity (different $K_S$), but form complexes with a (substrate analogous) inhibitor-molecule with (nearly) equal avidity (equal $K_I$).

It is much simpler and equally well in accordance with experimental results to suppose that only one reaction starting from para-aminobenzoic acid is inhibited by sulfanilamide. The apparent different para-aminobenzoic acid sensitivity of the various enzymes is then explained by a different avidity for the endproduct (X) of the inhibited reaction. The reactions between X and the apoenzymes $A_1$, $A_2$, and $A_3$ have different dissociation constants. The complete enzymes $E_1$, $E_2$ and $E_3$ catalyse the synthesis of methionine, xanthine and thymine respectively.

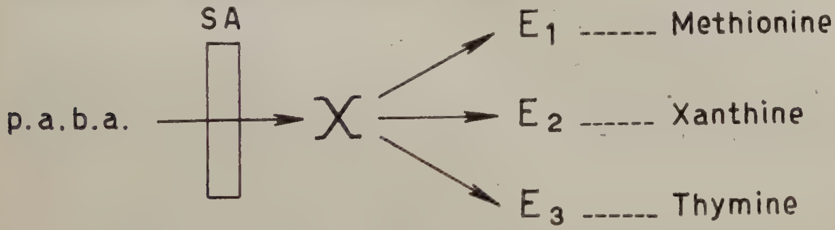

Fig. 3.

Many other arguments support the supposition, that sulfanilamide generally inhibits only one reaction (2).

The above concept is quite compatible with the conclusions, derived from the MICHAELIS-MENTEN equation. One has only to suppose that the enzymes catalyzing the synthesis of methionine, xanthine and thymine from their precursors have varying dissociation constants for their prosthetic group X.

We showed already that X was probably a folic acid derivative but not folic acid itself (DE HAAN (2)). Recently we found that the hypothetical intermediate X is not identical to the *Leuconostoc citrovorum* factor described by KERESZTESY and SILVERMANN (4).

## Summary.

Methionine, xanthine and thymine replace para-aminobenzoic acid as a growth factor for the para-aminobenzoic-less *E.coli* mutant strain 273.

The same substances were able to antagonize non competitively the bacteriostatical activity of 2000 mg/l sulfanilamide completely. It was further shown that the observed differences in sensitivity of the reaction chains leading to methionine, xanthine and thymine are not due to a difference in sensitivity to sulfanilamide but a difference in sensitivity to para-aminobenzoic acid. This seems to indicate that there exists only one reaction which is inhibited by sulfanilamide.

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# STUDIES ON HAEMAGGLUTINATION BY THE EMC-MM-COLUMBIA SK-GROUP OF VIRUSES

## I. FACTORS INFLUENCING THE HAEMAGGLUTINATION REACTION

by

**P. DE BAAN, J. D. VERLINDE and P. WALLER-FETTER**

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It has been shown by several investigators (HALLAUER (5), BREMER and MUTSAARS (2), VERLINDE and DE BAAN (9), OLITSKY and YAGER (8), GARD and HELLER (3), GASTAMBIDE-ODIER (4)), that the encephalomyocarditis, MM and Columbia SK viruses are capable to agglutinate sheep red cells, and that the GDVII strain of THEILER's mouse encephalomyelitis virus is capable to agglutinate human O erythrocytes (LAHELLE and HORSFALL (7)). In addition, DE BAAN (1) obtained agglutination of sheep red cells with virus strains called "High Lansing P/S II" and "High Lansing SCHULTZ", and agglutination of human O erythrocytes with the FA strain of THEILER's mouse encephalomyelitis virus. The so-called high Lansing strains are immunologically closely related to the group of the EMC, MM and Columbia SK viruses, and different from the original mouse-adapted Lansing strain of human poliomyelitis virus. The Lansing strain with which BREMER and MUTSAARS (2) produced haemagglutination appeared to be a virus of the EMC-MM-Columbia SK-group. In a previous paper (9) we mentioned a few weakly positive results with the original Lansing strain, but in repeated tests no haemagglutination with the Lansing strain and with the immunologically related Yale SK strain of human poliomyelitis virus could be produced (1).

No agglutination of sheep and human O erythrocytes occurred with the viruses of St. Louis encephalitis, the eastern and western type of the equine encephalomyelitis virus, the virus of lymphocytic choriomeningitis and rabies fixed virus.

In our previous paper a report is given of haemagglutination reactions carried out with centrifuged suspensions of fresh mouse brain and fresh sheep red cells, both diluted with saline. The present paper deals with the results of the haemagglutination reactions under varying conditions of the reagents. The preparation of the stock mouse brain and sheep red cell suspensions is described earlier (9, 1).

### 1. Haemagglutination reactions with erythrocytes of several sheep.

When the same virus suspension was tested against red cells of several sheep, a difference in haemagglutination titre was frequently observed (table 1). The lowest titres, and even negative reactions were obtained with erythrocytes of ewes, but when using cells of male sheep there were also considerable differences.

TABLE 1.

Haemagglutination titres with erythrocytes of 3 different sheep.

| Virus | Number of sheep | Haemagglutination titre | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| Col. SK | 1 | + | + | + | + | + | — | — |
| | 2 | + | + | + | + | — | — | — |
| | 3 | + | + | + | — | — | — | — |
| MM | 1 | + | + | + | + | + | — | — |
| | 2 | + | + | + | + | — | — | — |
| | 3 | + | + | — | — | — | — | — |

### 2. The age of the erythrocytes.

The highest titres were obtained with fresh red cells. The haemagglutination decreased in proportion to the duration of storage of the erythrocytes at 4° C. After 7 days of storage the titre was approximately one half of the titre obtained with the same cells in a fresh state. If only one animal is available, bleeding has to take place once a week. According to Gastambide-Odier (4), however, red cells from sheep bled every 15 days are useless for the haemagglutination reaction. The intervals between the bleedings should be longer.

### 3. The concentration of the erythrocyte suspension.

Haemagglutination reactions were carried out with 0.25, 0.5 and 1 per cent red cell suspensions. No striking differences in haemagglutination titres were observed, but after shaking and resettling of the agglutinated cells, the pattern of the 0.5 and 1 per cent suspensions was not as clear as that of the 0.25 per cent suspension. Consequently the latter, or even lower concentrations (0.1 per cent) are the most favourable ones for reading the result of the reaction.

### 4. The shape of the agglutination tubes.

When using the same virus and red cell suspensions, higher titres were obtained in long and narrow tubes, than in short and broad ones. This difference may be explained by assuming that the erythrocytes on their way to the bottom of the long tubes are in longer and more intensive contact with the virus particles, which facilitates adsorption of the virus to the cells and clumping of the cells.

### 5. The influence of the temperature.

When reading the result of the haemagglutination reaction within one hour, no striking difference in titre is observed at temperatures ranging from 4° C. to 35° C. Differences appear, however, if the result is read after longer periods, as is indicated in table 2.

TABLE 2.

Columbia SK haemagglutination reaction read after 4 hours at different temperatures.

| Temperature | Haemagglutination titre | | | | | |
|---|---|---|---|---|---|---|
| | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| 4° C. | + | + | + | + | + | + |
| 21° C. | + | + | + | + | — | — |
| 35° C. | + | + | ± | — | — | — |

### 6. The composition of the diluents.

According to Gard and Heller (3) optimal results are obtained when sheep blood is taken in an equal volume of Alsever's solution, and the red cells are washed and suspended in veronal-saline to a concentration of 0.1 per cent. Twofold dilutions of centrifuged mouse brain suspension are made with sheep brain extract. According to JUNGEBLUT (6) Krebs (mammalian Ringer) solution used as diluent for mouse brain would increase the heamagglutination titre. This has been confirmed, as is indicated in table 3. In addition, we found that allantoic fluid harvested from normal 10 to 12 days incubated chick embryos may also increase the haemagglutination titre.

TABLE 3.

Effect of diluents on the haemagglutination titre.

| Virus | Diluent | Haemagglutination titre | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| Col.SK | saline | + | + | + | — | — | — | — |
| | Krebs solution | + | + | + | + | — | — | — |
| | allantoic fluid | + | + | + | + | — | — | — |

## 7. Influence of pH.

It appeared from 11 repeated titrations at pH ranging from 4 to 9, that no striking differences in haemagglutination titres occurred.

## 8. Influence of storage of mouse brain.

The best results were generally obtained with freshly and aseptically removed brains of mice killed when paralysis was fully developed after intracerebral inoculation of virus. In mice that died, a rapid autolysis of the brain occurs, and autolyzed brain proved useless for the haemagglutination reaction. Likewise no haemagglutination could be produced with freshly removed brain after storage at -20° C without glycerol. When stored at -20° C. in 50 per cent glycerol, however, the haemagglutinating capacity was preserved for several months.

Centrifuged 5 per cent mouse brain suspensions generally maintained their haemagglutinating capacity only for a few days when stored at -20° C. In some suspensions, however, a decrease of the haemagglutination titre was noted. Perhaps the repeated freezing and thawing is responsible for the decrease in titre. It appears from table 4, that storage at higher temperatures is harmful for the haemagglutinating capacity.

TABLE 4.
Storage of centrifuged virus suspensions at various temperatures.

| Columbia SK mouse brain suspension | | Haemagglutination titre | | | | | |
|---|---|---|---|---|---|---|---|
| age | stored at | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| 24 hours | -20° C. | + | + | + | + | — | — |
| ,, | 4° C. | + | + | — | — | — | — |
| ,, | 21° C. | — | — | — | — | — | — |
| ,, | 37° C. | — | — | — | — | — | — |
| 48 hours | -20° C. | + | + | + | + | — | — |
| ,, | 4° C. | + | — | — | — | — | — |
| ,, | 21° C. | + | + | — | — | — | — |
| ,, | 37° C. | — | — | — | — | — | — |
| 72 hours | -20° C. | + | + | + | — | — | — |
| ,, | 4° C. | — | — | — | — | — | — |
| ,, | 21° C. | — | — | — | — | — | — |

### 9. Influence of ultraviolet irradiation.

Columbia SK virus in 10 per cent mouse brain suspensions was partly inactivated by ultraviolet irradiation, so that the mouse infectivity titre as measured by intracerebral inoculation, decreased from $10^{-10}$ to $10^{-2}$. Notwithstanding the considerable decrease of virulence, the haemagglutination titre was maintained at the same level as that of the original suspensions. Completely inactivated 1 per cent suspensions also appeared to maintain their haemagglutinating ability.

### 10. Experiments with filtrated virus suspensions.

Columbia SK-infected as well as normal mouse brain suspensions produced high haemagglutination titres after filtration of the centrifuged suspensions through a Seitz EK filter. The titres were low or negative when using fresh filtrates, but a considerable increase in titre was noted after storage of the filtrate at -20° C. for several days. As can be seen in figure 1, the highest titres were obtained after 4 to 5 days storage.

Haemagglutination of normal mouse brain suspensions was only produced after filtration through Seitz filter, but not after filtration through "Cella" filters.

Consequently an agglutinin is present in normal mouse brain, that exerts its influence in Seitz-filtered suspensions. Presumably a haemagglutination inhibiting substance is also present in mouse brain, which is partly adsorbed by the Seitz filter, but not by the Cella filter, and which is partly destroyed by freezing, as a consequence of which the haemagglutination titre increases during the first few days of storage at -20° C.

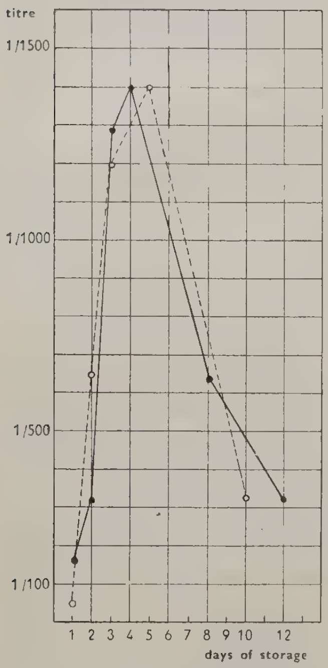

Fig. 1. Haemagglutination of filtered mouse brain suspensions after storage at –20° C.

———— Columbia SK-infected mouse brain.

– – – – – normal mouse brain.

The presence of an inhibitor in normal mouse brain is demonstrated in the following experiment.

Two series of twofold dilutions of a Seitz filtrate of normal mouse brain suspension were prepared. A 5 per cent centrifuged normal mouse brain suspension was added to all tubes of the second series, in such a way that the final concentrations of mouse brain filtrate in the corresponding tubes of both series were the same. Both series were placed at 37° C. in a waterbath for 20 minutes, and after cooling to room temperature equal parts of a 0.25 per cent suspension of sheep erythrocytes were added to each tube. Table 5 shows that the centrifuged normal mouse brain suspension produced a 16-fold decrease of the haemagglutination titre, which may de due to an inhibitory activity of the unfiltered mouse brain suspension.

With regard to the haemagglutination by Columbia SK virus no clear-cut results were obtained as to the inhibitory activity of centrifuged normal mouse brain suspensions, whereas the agglutination of fowl red cells by influenza A virus was strongly inhibited (table 6).

TABLE 5.

The influence of centrifuged normal mouse brain suspension on the haemagglutinating capacity of a Seitz filtrate of normal mouse brain suspension.

| normal mouse brain suspension | Haemagglutination titre | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| Seitz filtrate | + | + | + | + | + | + | — |
| centrifuged suspension | — | — | — | — | — | — | — |
| centrifuged suspension added to a Seitz filtrate | + | + | — | — | — | — | — |

TABLE 6.

Influence of a haemagglutination inhibitor in normal mouse brain on the haemagglutination by Columbia SK and influenza A viruses.

| Erythrocytes | Virus | Diluent | Haemagglutination titre | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| sheep | Col. SK | saline | + | + | + | + | + | + |
| ,, | ,, | mouse brain suspension | + | + | + | + | + | — |
| fowl | Influenza | saline | + | + | + | + | + | — |
| ,, | ,, | mouse brain suspension | + | — | — | — | — | — |

The difference might be explained by assuming that the Columbia SK-haemagglutination is inhibited beforehand by the inhibitor already present in the infected mouse brain, whereas allantoic fluid may contain much less of inhibiting substance. This has been shown in an experiment in which allantoic fluid did not markedly inhibit the agglutination of sheep and fowl red cells by Columbia SK virus and influenza A virus respectively (table 7).

On the contrary, when testing the effect of diluent (saline, Krebs solution, and allantoic fluid) on the Columbia SK haemagglutination titre, we found an increase rather than a decrease in titre.

The possibility that the non-specific haemagglutinin in normal mouse brain might be a heterophilic antibody, and that the inhibitor

TABLE 7.

Effect of normal allantoic fluid on the haemagglutination by Columbia SK and influenza A viruses.

| Erythrocytes | Virus | Diluent | Haemagglutination titre | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| sheep | Col.SK | saline | + | + | + | + | + | — |
| ,, | ,, | allantoic fluid | + | + | + | + | + | — |
| fowl | Influenza | saline | + | + | + | + | + | — |
| ,, | ,, | allantoic fluid | + | + | + | + | ± | — |

might be related to FORSSMAN's antigen, which is present in mice, could not be excluded.

In order to test this hypothesis, attempts were made to absorb the non-specific haemagglutinin present in filtrates of normal mouse brain by adding guinea pig kidney.

Equal parts of filtered mouse brain suspension and a thick suspension of minced guinea pig kidney were mixed and placed at 4° C. for 1 to 2 hours. After centrifuging, twofold dilutions of the supernatant fluid were mixed with a 0.25 per cent suspension of sheep erythrocytes. A series of the original filtrate with corresponding dilutions was used as control.

It appears from table 8, that guinea pig kidney removes the non-specific haemagglutinin, as it absorbs heterophilic antibody.

TABLE 8.

Absorption of non-specific haemagglutinin in normal mouse brain by guinea pig kidney.

| normal mouse brain filtrate | Haemagglutination titre | | | | | |
|---|---|---|---|---|---|---|
| | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 |
| without treatment | + | + | + | + | — | — |
| after treatment with guinea pig kidney | — | — | — | — | — | — |

This, however, is not a proof for the identity of the non-specific haemagglutinin with heterophilic antibody. As a matter of fact, the non-specific haemagglutinin is unstable at 4° C., since control filtrates lose their haemagglutinating ability within 12 hours. The heterophilic antibody, on the contrary, is much more stable. Therefore, we are inclined to consider the absorbing activity of guinea pig kidney as a result rather of the presence of an inhibitor similar to the inhibitor in mouse brain, than of FORSSMAN's antigen.

## Summary and conclusions.

A number of factors may exert a favourable or an inhibitory effect on the haemagglutination of sheep erythrocytes by Columbia SK and related viruses. Concerning the erythrocytes, evidence of individual differences of the sheep has been obtained. Moreover, the age of the red cell is important in so far, that fresh cells give better results than cells stored at 4° C. for one week. The intervals at which the sheep are bled should not be too short. The concentration of the erythrocytes should not be higher than 0.25 per cent and clear readings are obtained after 2 to 4 hours at 4° C. When Krebs solution or normal allantoic fluid are used as diluent instead of saline, a higher titre may be read. Haemagglutination occurs when freshly removed brains of mice killed at the paralytic stage of the experimental disease are used immediately or after storage in glycerol in the frozen state. Centrifuged brain suspensions maintain their specific haemagglutinating ability only for a few days when stored at -20° C. No correlation could be demonstrated between the mouse infectivity and the haemagglutination titre. Frozen suspensions may lose their haemagglutinating ability within a couple of weeks, but the mouse infectivity titre, though gradually decreasing, may remain high for several weeks. On the other hand, the haemagglutinating ability may be maintained when the mouse infectivity is decreased or completely lost by ultraviolet irradiation.

Non-specific haemagglutination occurs with normal mouse brain suspensions when filtrated through Seitz filters and stored at -20° C. Since the non-specific haemagglutinin cannot be demonstrated in centrifuged suspensions, it is suggested that it is masked by an inhibitor present in normal mouse brain, which may be partly adsorbed by the Seitz filter, and partly destroyed by freezing.

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# STUDIES ON HAEMAGGLUTINATION BY THE EMC-MM-COLUMBIA SK-GROUP OF VIRUSES

## II. THE IDENTIFICATION OF VIRUSES AND ANTIBODIES BY THE HAEMAGGLUTINATION-INHIBITION REACTION[1]

by

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(Received December 7, 1950).

When rabbits and monkeys are immunized by intramuscular injections of 1 ml of a 10 per cent suspension of virulent mouse brain, or when monkeys survived the intracerebral inoculation with viruses of the EMC-MM-Columbia SK group, antibodies inhibiting the haemagglutination reaction can be demonstrated in their serum, as has been shown by VERLINDE and DE BAAN (7), BREMER and MUTSAARS (2), DE BAAN (1), GARD and HELLER (3). When carrying out the haemagglutination-inhibition reaction, it may be noted, that in the tubes containing the lower serum dilutions, agglutination of the erythrocytes may occur as a result of the presence of heterophilic antibody in the serum. In rabbits, the titre of heterophilic antibody, as a rule, does not exceed 1/16; in monkeys this titre may be as high as 1/32 or 1/64. When the titre of haemagglutination-inhibiting antibodies is higher than the heterophilic antibody titre, and the reaction is carried out with a constant number of haemagglutinating units, no haemagglutination is observed in a certain number of tubes following those in which haemagglutination due to heterophilic antibody occurs, until the dilution of serum is reached, in which the amount of haemagglutination-inhibiting antibodies is too low to inhibit clumping of erythrocytes. The highest dilution of serum which prevents haemagglutination is the haemagglutination-inhibition titre.

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[1] First publication: Antonie van Leeuwenhoek **17**, 119, 1950.

Haemagglutination reactions were carried out with sera from rabbits and monkeys, which had been immunized against the viruses of the EMC-MM-Columbia SK-group, against the Lansing type and against a rodent-nonpathogenic strain of human poliomyelitis virus isolated in this laboratory from the stools of a patient suffering from poliomyelitis (strain Rieth.A.K.1949, VERLINDE and NIHOUL (9)). Moreover seven sera of rhesus monkeys immunized against Yale SK virus, and 10 human poliomyelitis convalescent sera sent by Dr C. W. JUNGEBLUT, New York, were examined. The results are presented in table 1.

It appears from table 1, that no doubt can exist about the close antigenic relationship between the members of the EMC-MM-Columbia SK-group, in which group the so-called "High Lansing" strains PS/II and SCHULTZ also have to be grouped. All animals, except Rhesus 28 have developed haemagglutination-inhibiting antibodies to this group of viruses. The results of the haemagglutination-inhibition reactions are in agreement with those of the neutralization tests carried out by others.

Table 1 also shows, that the immune sera to the Lansing-Yale SK type of human poliomyelitis virus fail to inhibit the haemagglutination by Columbia SK virus, except two monkey sera of which one (Cynomolgus 37) has been immunized with Yale SK virus in this laboratory, and one with Yale SK virus by DR C. W. JUNGEBLUT, New York. The other rhesus monkeys (marked AT and AS) have also been immunized by DR JUNGEBLUT with the same strain, without developing haemagglutination-inhibiting antibodies. Some of them, however, possessed antibodies neutralizing Columbia SK virus [1]). This virus was also partly neutralized by some of 10 human poliomyelitis convalescent sera [1]), one of which had developed haemagglutination-inhibiting antibodies.

Since no antigenic relationship between Columbia SK and Yale SK virus has been demonstrated by neutralization tests (MELNICK (5) it is difficult to accept, that the Columbia SK antibodies in the two monkeys have developed as a result of the immunization with Yale SK virus. It seems more likely to consider the possibility of a preceding incidental infection of the two monkeys as well as a similar infection of patient 1 with a virus of the EMC-MM-Columbia SK-group, as a result of which the antibodies have developed.

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[1]) C. W. JUNGEBLUT, Personal communication.

TABLE 1.

Haemagglutination-inhibition reactions using 4 H.U. of virus.

| | | Haemagglutination-inhibition titre | | | | |
|---|---|---|---|---|---|---|
| Immune serum | Virus: | Col. SK | MM | EMC | ,,High Lansing" strains | |
| | | | | | PS/II | Schultz |
| Col.SK (rabbit) | | 2048 | 512 | 2048 | 8192 | 16384 |
| Col.SK (Cynomolgus 41, after i.c. inoculation) | | 4096 | | | | |
| Col.SK (Cyn. 41 after 3 subsequent intramuscular injections) | | 16384 | | | | |
| Col.SK (Rhesus 28, after i.c. inoculation) | | 0 | | | | |
| Col.SK (Rhesus 33, after i.c. inoculation) | | 512 | | | | |
| Col.SK (Cyn. 102, after 1 intramuscular injection) | | 4096 | | | | |
| Col.SK (Cyn. 102, after 4 intramuscular injections) | | 32768 | | | | |
| MM (rabbit) | | 512 | 256 | 1024 | | |
| EMC (rabbit) | | 8192 | 1024 | 4096 | | |
| "High Lansing" Schultz (rabbit) | | 8192 | | | 8192 | 8192 |
| Lansing (rabbit) | | 0 | 0 | | | |
| Lansing (monkey) | | 0 | 0 | | | |
| Yale SK (rabbit) | | 0 | 0 | | | |
| Yale SK (Cynomolgus 37) | | 512 | | | | |
| Yale SK (Rhesus AT 4) | | 0 | | | | |
| Yale SK (Rhesus AT 6) | | 0 | | | | |
| Yale SK (Rhesus AT 7) | | 0 | | | | |
| Yale SK (Rhesus AT 8) | | 0 | | | | |
| Yale SK (Rhesus AS 77) | | 0 | | | | |
| Yale SK (Rhesus AS 79) | | 128 | | | | |
| Yale SK (Rhesus AS 80) | | 0 | | | | |
| Rieth.A.K.1949 (Cynomolgus 38) | | 0 | | | | |
| Patient 1 (poliomyelitis convalescent) | | 128 | | | | |
| ,, 2 ( ibid. ) | | 0 | | | | |
| ,, 3 ( ibid. ) | | 0 | | | | |
| ,, 4 ( ibid. ) | | 0 | | | | |
| ,, 5 ( ibid. ) | | 0 | | | | |
| ,, 6 ( ibid. ) | | 0 | | | | |
| ,, 7 ( ibid. ) | | 0 | | | | |
| ,, 8 ( ibid. ) | | 0 | | | | |
| ,, 9 ( ibid. ) | | 0 | | | | |
| ,, 10 ( ibid. ) | | 0 | | | | |

It has been shown, that antibodies to the EMC-MM-Columbia SK group of viruses indeed may occur in man. In Holland we have not been able thus far to demonstrate these antibodies in human sera either from poliomyelitis convalescents or from others. In the U.S.A., however, JUNGEBLUT (4) found in 1948—1949 21 per cent of 126 human poliomyelitis convalescent sera neutralizing Columbia SK virus, and GARD and HELLER (3) reported from Sweden, that 25 per cent of 171 sera from patients suffering from disorders of the central nervous system, and 13.6 per cent of normal human sera inhibited the haemagglutination by MM virus. The highest indices, ranging from 8 to 64, were found in 23 (13.4 per cent) of the 171 sera from patients suffering from central nervous system diseases, among which 12 cases of poliomyelitis. In only one (2.3 per cent) of the 44 normal sera an inhibition index of 8 to 16 was found.

Like in sera against the Lansing-Yale SK type of human poliomyelitis virus, no haemagglutination-inhibiting antibodies were found in sera from convalescent monkeys infected with two virus strains fulfilling the definition of human poliomyelitis virus (strains Rieth. A.K.1949 and Rotterdam W.H.1950). Likewise, no haemagglutination-inhibition occurred with sera from rabbits immunized against the viruses of eastern and western equine encephalomyelitis, St. Louis encephalitis, lymphocytic choriomeningitis and rabies (fixed virus).

It seems that sufficient evidence has been obtained, that the viruses of the EMC-MM-Columbia SK group and antibodies to these viruses may be identified by the haemagglutination-inhibition reaction. The advantage of a rapid identification of a virus belonging to this group by the simple haemagglutination-inhibition test has been shown in two recent accidental laboratory infections of mice with Columbia SK virus.

The first example is that of contamination of a strain of Japanese B encephalitis virus with Columbia SK virus.

In a previous paper (7), we mentioned that the Nakayama strain of the virus of Japanese B encephalitis agglutinated sheep red cells, and that the haemagglutination was inhibited by homologous immune serum as well as by immune sera prepared against Columbia SK and MM virus, thus indicating an immunological relationship between Japanese B encephalitis virus and the EMC-MM-Columbia SK-group, which had never been shown by neutralization tests.

When Dr A. B. Sabin, Cincinnati, after a visit to this laboratory, repeated our experiments, it was found that the virus of Japanese B encephalitis was indeed associated with a specific haemagglutinin for sheep and chick red cells, and that regular and reproducible results were obtained particularly when using erythrocytes from chicks bled during the first 24 hours after hatching (Sabin and Buescher (6)). This haemagglutinin, however, was found to possess properties different from all other viral haemagglutinins described thus far, so that it became evident, that we had not been working with pure Japanese B encephalitis virus, but with a virus accidentally contaminated with one of the viruses of the EMC-MM-Columbia SK group.

That this conception was right, has been shown in animal experiments, and in neutralization and haemagglutination-inhibition reactions with the Okinawa strain of Japanese B encephalitis virus and human serum containing antibodies to that strain, both kindly provided by Dr Sabin. Unfortunately we did not succeed in producing a haemagglutination titre with erythrocytes from sheep and from newly hatched chicks high enough for carrying out inhibition reactions. The Japanese B immune serum, however, constantly failed to inhibit the haemagglutination by Columbia SK virus, and to neutralize this virus in the mouse protection test. The immune serum prepared to the virus, which we had erroneously labelled Japanese B encephalitis virus, however, neutralized Columbia SK virus completely, and appeared to possess antibodies inhibiting the haemagglutination by Columbia SK virus to a titre of 512.

The identification of this virus in the experimental animal presented more difficulties. Mice are not suitable for the differentiation between Japanese B encephalitis virus and Columbia SK virus, since encephalomyelitis may develop after intracerebral and peripheral inoculation of both viruses. Monkeys are susceptible to the virus of Japanese B encephalitis, but *Macacus cynomolgus* proved, in our hands (Verlinde, de Baan and Vercruysse (8)), also susceptible to the Columbia SK virus. Kittens could not be infected with both viruses, and a young sheep, which is said to be susceptible to the virus of Japanese B encephalitis, did not show any signs of illness after the intracerebral inoculation of the questionable virus. In rabbits and cynomolgus monkeys antibodies strongly neutralizing the questionable virus and Columbia

SK virus, developed. In the serum of these animals also antibodies inhibiting the Columbia SK haemagglutination could be shown. Remarkably in the infected sheep no haemagglutination-inhibiting antibodies could be demonstrated, although antibodies neutralizing the Columbia SK virus were present to a high titre.

The second example of a possible laboratory infection of inoculated mice with Columbia SK virus occurred when suckling mice were inoculated with centrifuged stool suspensions treated with penecillin and streptomycin. The stools were sampled from patients suffering from minor illness, frequently associated with meningeal symptoms. They were examined for the presence of poliomyelitis virus by inoculation of cynomolgus monkeys, and for the presence of Coxsackie virus by inoculation of suckling mice. Among many negative stool samples, that of one patient caused death in suckling mice two days after inoculation, and in subsequent passages, in which the animals died with paralysis invariably after two days, a virus was isolated. This virus, however, did not produce an extensive myositis in suckling mice only, but also in adult mice after intramuscular inoculation. Moreover, it produced paralysis in adult mice after intracerebral and intraperitoneal inoculation, and it caused haemagglutination of sheep erythrocytes. Since we found, that this haemagglutination was specifically inhibited by Columbia SK monkey immune serum and by EMC rabbit immune serum to a titre of 8192, there seems to be no doubt, that the virus is identical with the viruses of the EMC-MM-Columbia SK-group. In the acute phase and convalescent serum of none of the patients that suffered from the disease, the patient with the "positive" stool sample included, antibodies neutralizing this virus, or inhibiting its haemagglutination reaction could be shown, so that an accidental infection of the very first suckling mouse passage has to be accepted.

## Summary and conclusions.

The haemagglutination-inhibition reaction revealed a close antigenic relationship between the viruses of the EMC-MM-Columbia SK-group. Though it has been shown by haemagglutination-inhibition reactions, that this group is not related to the Lansing-Yale SK type and other types of human poliomyelitis virus, two of eight monkeys immunized with Yale SK virus appeared to possess antibodies inhibiting the haemagglutination by Columbia SK virus. This observation is considered to be due to a preceding incidental infection with a virus of the EMC-MM-Columbia SK-group rather than to an antigenic overlapping of this group and the Lansing type. Laboratory infections of animals with viruses of the EMC-MM-Columbia SK group appeared to be not uncommon. In two cases, a laboratory infection of mice with viruses of this group could be traced by identification of the viruses by means of the haemagglutination and haemagglutination-inhibition reaction. The haemagglutination reaction by Columbia SK virus is not inhibited by immune sera against the viruses of Japanese B encephalitis, St. Louis encephalitis, eastern and western equine encephalomyelitis, lymphocytic choriomeningitis and rabies (fixed virus).

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A. Schierbeek. Antonie van Leeuwenhoek. Zijn leven en werken (Antonie van Leeuwenhoek. His life and his work. 2 Vols) — de Tijdstroom, Lochem. 1950.

As the editing of the complete Series of Letters of Leeuwenhoek will still cover many years, Schierbeek, the Editor in Chief of these Letters has undertaken the composition of a book, which by its more restricted content will appeal to a greater and more varied number of readers. The first volume opens with a biography of Leeuwenhoek. In further separate chapters his relation to the science of the inorganic world, of microbiology, of lower animals, of entomology is exposed. The second volume will contain chapters on his studies of the vertebrates, his views on the doctrine of generation, on histology and physiology, on the medical science of his time, on botany, on ecology.

As Leeuwenhoek has generally re-taken up his subjects after wide intervals of time, it is hard to arrive at an all round view of his conceptions, so the gathering of observations and considerations, bearing on a same subject but scattered over many letters, is very helpful to the reader. For microbiology this work has been already accomplished masterly by Dobell in Antony van Leeuwenhoek and ,,his little animals'' and the author over and again cites Dobell with the greatest appreciation. The present work, however, has a wider scope, and the work carried on by Leeuwenhoek in many other fields is of great interest too. It is moreover a pleasure for his countrymen to read the writings of Leeuwenhoek in the original language.

The author takes a very definite stand against those who limit the genius of Leeuwenhoek to the presence of a remarkable power of observation and most skilful hands. Over and again Schierbeek lays stress on the scientific mode of experimental approach, the scientific doubt, the very distinct line drawn by Leeuwenhoek between facts ascertained by him or merely imagined, his bland acknowledgement of former errors.

Also those who have greatly enjoyed Dobell's ,,little animals'' will find fresh pleasure in this stimulating book of one of our countrymen.

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# STUDIES ON HAEMAGGLUTINATION BY THE EMC-MM-COLUMBIA SK-GROUP OF VIRUSES

## III. DESTRUCTION OF VIRUS RECEPTORS IN VITRO AND IN VIVO [1]

by

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(Received January 19, 1951).

Fowl red cells become inagglutinable for the viruses of the mumps-Newcastle disease-influenza group after adsorption and subsequent elution of these viruses. The elution is considered as a result of destruction of virus receptors on the surface of the red cells according to the receptor gradient (BURNET, MCCREA and STONE (3)). Destruction of virus receptors also occurs when the erythrocytes are treated with a filtrate of a culture of *Vibrio cholerae* (MCCREA (4)). A receptor destroying enzyme (RDE) is thought to be responsible for the destruction of virus receptors. Cholera filtrate has been shown effective in destroying virus receptors of the isolated mouse lung (FAZEKAS DE ST. GROTH (5)) and of the chorioallantoic membrane of the chick embryo (STONE (8)), thus preventing the adsorption of influenza virus to the living cell.

In a preliminary note (9) destruction of the receptors for the Columbia SK virus of sheep erythrocytes and in the living mouse has been reported. Additional data are presented in this paper.

### Adsorption of virus to sheep erythrocytes.

In 6 tubes a series of twofold dilutions of brain suspension of mice inoculated intracerebrally with Columbia SK virus was mixed with equal volumes of a 0.25 per cent suspension of washed sheep red cells, the total volume in each tube being 4 ml. The tubes were

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[1] Second publication: Antonie van Leeuwenhoek **17**, 129, 1951.

placed at 4° C., and after 5 and 10 hours, when haemagglutination was observed to a titre of 1/160, 0.5 ml of the clear supernatant fluid was examined for haemagglutinating ability.

It appears from table 1, that the haemagglutinating ability of the supernatant fluid has decreased proportionally to the concentration of virus originally present in each tube, which indicates that the virus has been adsorbed to the red cells.

Table 1.

Adsorption of Columbia SK virus to sheep red cells.

| | | Haemagglutination titre | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| Fresh original suspension | | + | + | + | + | + | — |
| After 5 hours | original suspension | + | + | + | + | + | — |
| | supernate tube 1 | + | + | + | — | — | — |
| | ,, ,, 2 | + | + | — | — | — | — |
| | ,, ,, 3 | + | — | — | — | — | — |
| | ,, ,, 4 | — | — | — | — | — | — |
| | ,, ,, 5 | — | — | — | — | — | — |
| After 10 hours | original suspension | + | + | + | + | — | — |
| | supernate tube 1 | + | + | — | — | — | — |
| | ,, ,, 2 | + | + | — | — | — | — |
| | ,, ,, 3 | + | — | — | — | — | — |
| | ,, ,, 4 | — | — | — | — | — | — |
| | ,, ,, 5 | — | — | — | — | — | — |

### Elution.

Two mixtures of equal volumes of a 5 per cent brain suspension of mice inoculated intracerebrally with Columbia SK virus, and a 50 per cent suspension of washed sheep red cells were placed overnight at 4° C. After centrifuging at low speed part of the supernate was tested, and proved completely devoid of haemagglutinating power, as a result of adsorption of the virus. The tubes were then shaken thoroughly, and one was placed in a waterbath at 37° C., and the other at 4° C. After 1, 2, 3, 4 and 6 hours the mixtures were centrifuged at low speed, and the supernatant fluid was tested for haemagglutinating power.

It appears from table 2, that partial elution of the virus has occurred in the tubes kept at 37° C. for at least 1 hour, whereas no elution was demonstrated after the same time intervals at 4° C. OLITSKY and YAGER (7) also found elution at 37° C.

Table 2.

Elution of Columbia SK virus.

| | Haemagglutination titre | | | | |
|---|---|---|---|---|---|
| | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 |
| Original virus overnight at 4° C. | + | + | + | + | — |

| | Tube kept at 37° C. | | | | | Tube kept at 4° C. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 |
| 1 hour | + | — | — | — | — | — | — | — | — | — |
| 2 hours | + | — | — | — | — | — | — | — | — | — |
| 3 hours | — | — | — | — | — | — | — | — | — | — |
| 4 hours | ± | ± | — | — | — | — | — | — | — | — |
| 6 hours | — | — | ± | — | — | — | — | — | — | — |

A complete elution was not obtained, even when the tubes were kept at 37° C. overnight. Therefore, it was not possible to test whether or not the red cells would have lost their agglutinability.

In a recent paper, however, BREMER (10) reports a quick and complete elution at 37° C., after which the erythrocytes have maintained their agglutinability by the same virus.

## Destruction of virus receptors of sheep red cells.

No evidence of receptor destruction by the virus itself could be obtained because of the incomplete elution of the virus at 37° C.

In filtrates of cultures of *Vibrio cholerae* prepared according to the method of BURNET (2) and slightly modified as described by DE BAAN (1), a receptor destroying agent could be demonstrated.

The receptor destroying activity of the filtrates was determined quantitatively by mixing 0.25 ml of twofold dilutions of filtrate, and 0.25 ml of a 0.5 per cent suspension of sheep erythrocytes. The tubes were placed in a waterbath at 37° C. for 30 minutes. Then, 0.5 ml of a dilution of Columbia SK virus infected mouse brain suspension representing 4 to 16 haemagglutinating units was added, and the haemagglutination reaction was read after 2 to 4 hours at 4° C. An example of such a titration, in which the enzymatic activity proved to be 1/640 for MM, and 1/1280 for Columbia SK virus, is given in table 3.

Table 3.

Titration of receptor destroying enzyme (RDE).

| Virus | H.U. | Final dilutions of filtrate | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | 1/5120 |
| Col SK | 16 | — | — | — | — | — | — | — | + | + |
| MM | 16 | — | — | — | — | — | — | + | + | + |

It is of interest to note, that the RDE titres of different samples of cholera filtrate may vary considerably when titrated with the same virus and erythrocytes of the same sheep. Moreover, there is no parallelism between the RDE titre against Columbia SK and influenza virus. The titres against the former are often considerably higher than those against the latter virus.

### Destruction of virus receptors of the living cell.

In a previous paper we reported (9) to have obtained a certain resistance of mice against the intraperitoneal inoculation of Columbia SK virus after intraperitoneal injection of cholera filtrate. When mice were injected intraperitoneally with cholera filtrate and Columbia SK virus simultaneously, 40 per cent (22 of 56 animals) survived, and the others died 3 to 17 (average 8) days after infection. When infected mice were treated every day during 7 days, 75 per cent (18 of 24 animals) survived, and the others died 7 to 17 (average 12) days after infection. Only 2 of 30 non-treated control mice survived the infection, and the others died 4—10 (average 5) days after infection. Jungeblut (6), when repeating these experiments, obtained similar results.

In a second series of experiments, taken some months later, in the series of animals once injected with cholera filtrate 37 per cent, and in the series of animals injected 2 to 7 times, 46 per cent survived, whereas 94 and 100 per cent of the control animals died (tables 4, 5 and 6).

As a matter of fact, cholera filtrate seems to have a certain protective potency on the Columbia SK infection in the mouse, when injected simultaneously or some hours before the virus inoculation. The protective activity of the filtrate is shown by the survival of a certain percentage of the animals, which is significantly

Table 4.

Mice injected intraperitoneally with 0.5 ml of cholera filtrate in various dilutions, and Columbia SK virus (100 $\text{ID}_{50}$) simultaneously.

| | number of mice | | | average time of survival (days) |
|---|---|---|---|---|
| | injected | survived | died | |
| control mice | 16 | 0 | 16 | 5 |
| cholera filtrate undiluted | 4 | 2 | 2 | 4 |
| ,, ,, 1/10 | 16 | 5 | 11 | 7 |
| ,, ,, 1/100 | 16 | 4 | 12 | 6 |
| ,, ,, 1/1000 | 16 | 5 | 11 | 5 |

Table 5.

Mice injected intraperitoneally with 0.5 ml of cholera filtrate in a dilution 1/10, and varying dilutions of Columbia SK virus with intervals ranging from 5 minutes to 24 hours. The cholera filtrate was injected before the virus.

| interval | Number of mice | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10.000 $\text{ID}_{50}$ | | | 1000 $\text{ID}_{50}$ | | | 100 $\text{ID}_{50}$ | | |
| | inj. | died | average time of survival (days) | inj. | died | average time of survival (days) | inj. | died | average time of survival (days) |
| control | 4 | 4 | 6 | 4 | 4 | 5 | 24 | 22 | 6 |
| 5 min. | | | | | | | 36 | 22 | 7 |
| 30 ,, | 4 | 2 | 8 | 4 | 3 | 7 | 4 | 3 | 13 |
| 1 hour | 4 | 3 | 8 | 4 | 2 | 6 | 22 | 14 | 9 |
| 6 hours | | | | | | | 18 | 12 | 5 |
| 18 ,, | | | | | | | 18 | 16 | 7 |
| 24 ,, | | | | | | | 18 | 15 | 7 |

Table 6.

Mice injected intraperitoneally 2 to 7 times during one week with 0.5 ml of cholera filtrate in a dilution 1/10, and simultaneously with the first injection inoculated intraperitoneally with 100 $\text{ID}_{50}$ of Columbia SK virus.

| | number of mice | | | average time of survival (days) | % of survivors |
|---|---|---|---|---|---|
| | injected | survived | died | | |
| control mice | 20 | 1 | 19 | 5 | 5 |
| treated daily | 46 | 24 | 22 | 8 | 52 |
| treated every second day | 22 | 7 | 15 | 6 | 32 |
| treated every third day | 22 | 2 | 20 | 6 | 9 |

higher than that of the control mice of which almost 100 per cent of the animals come down, and by a more or less marked prolonged survival time of the mice that come down ultimately.

The experiments mentioned thus far have been carried out with filtrates prepared from the Inaba strain of *V. cholerae.* When testing the protective potency of filtrates prepared from various strains of *V. cholerae,* differences appeared (table 7). The strains used were kindly supplied by the Institute of Tropical Hygiene, Leiden (Prof. Dr J. E. DINGER). Groups of 10 mice were inoculated intraperitoneally with varying $ID_{50}$ of Columbia SK virus (mouse brain). Three hours prior to inoculation one half of the mice received 1 ml of undiluted, and one half received 1 ml of tenfold diluted cholera filtrate intraperitoneally. Control mice were infected, but not treated with cholera filtrate. Table 7 shows, that protection is obtained in the groups of mice infected with 10 and with 100 $ID_{50}$. Protection is evident by the prolonged average time of survival and by the percentage of survivors, which is highest in the animals treated with filtrate of the Inaba strain.

Table 7.

Protection by filtrates of various strains of *Vibrio cholerae.*

| strain of *V. cholerae* | 1000 $ID_{50}$ | | | | 100 $ID_{50}$ | | | | 10 $ID_{50}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | inj. | died | average time of survival (days) | % of survivors | inj. | died | average time of survival (days) | % of survivors | inj. | died | average time of survival (days) | % of survivors |
| controls | 5 | 5 | 4 | 0 | 5 | 5 | 5 | 0 | 5 | 5 | 5 | 0 |
| Inaba | 10 | 10 | 5 | 0 | 10 | 7 | 9 | 30 | 10 | 5 | 6 | 50 |
| Ogawa | 10 | 9 | 4 | 10 | 10 | 9 | 6 | 10 | 10 | 8 | 7 | 20 |
| "45" | 10 | 10 | 5 | 0 | 10 | 10 | 5 | 0 | 10 | 9 | 6 | 10 |
| P24 | 10 | 10 | 6 | 0 | 10 | 8 | 5 | 20 | 10 | 6 | 5 | 40 |

### Mechanism of the action of cholera filtrate.

During the first series of experiments, it has been shown, that the virus itself is not attacked by the cholera filtrate. Therefore, 6 mice were injected intraperitoneally with 0.5 ml of cholera filtrate (Inaba strain) diluted 1 : 100, and 6 hours later with 0.5 ml mouse brain suspension representing 500 $LD_{50}$ of Columbia SK virus. Every 24 hours the same amount of cholera filtrate was administered intraperitoneally for 5 days. At 24 hour intervals one animal was killed, and washings of the abdominal cavity with 1 ml of saline were injected intracerebrally into 4 mice. In this experiment the virus could be shown in the abdominal cavity 24, 48, and 72 hours after the intraperitoneal inoculation, whereas in non-treated control mice the virus had completely disappeared from the abdominal cavity within 24 hours. These results strongly suggest that the virus, following inoculation, is readily adsorbed, and that the cholera filtrate may prevent the adsorption by enzymatic destruction of virus receptors, which are presumably present in the abdominal cavity.

Since a number of treated animals after a single injection come down, it is suggested that the filtrate does not always attack all the virus receptors present in the abdominal cavity, or that a rapid regeneration of virus receptors may occur as soon as the filtrate has disappeared from the abdominal cavity. The higher percentage of protection in groups of animals injected with filtrate every day or every second day seems to be in favour of this hypothesis.

On the other hand, the development of immunity in the temporarily protected mice has to be considered. When mice, however, that survived the Columbia SK virus infection together with the cholera filtrate treatment, were reinfected intraperitoneally, 47 per cent proved fully susceptible to reinfection, 11 per cent were partly, and 42 per cent proved completely immune to reinfection. Thus, in approximately one half of the mice treated with cholera filtrate, an actual protection, which has kept the virus outside the cells, and which is not due to active immunity, seems to have occurred. When the virus has not been adsorbed to the cells, it remains for a few days in active state in the abdominal cavity, and then dies off.

In the experiments reported, the cholera filtrate and the virus were administered by the same, intraperitoneal, route. When administering them by different routes, no definite protection was obtained (table 8). This also seems to be in favour of a destruction of virus receptors only there where the filtrate comes in contact with the cells to which the virus is adsorbed.

Table 8.

Administration of cholera filtrate and Columbia SK virus by different routes.

| Cholera filtrate | Col. SK virus | number of mice | | | average time of survival (days) |
|---|---|---|---|---|---|
| | | injected | survived | died | |
| subcut. 4 daily inj. of 1 ml | intraperit. | 12 | 0 | 12 | 6 |
| intraper. 4 daily inj. of 1 ml | intraperit. | 12 | 7 | 5 | 10 |
| no treatment | intraperit. | 6 | 0 | 6 | 6 |
| subcut. 4 daily inj. of 1 ml | orally | 12 | 3 | 9 | 7 |
| intraper. 4 daily inj. of 1 ml | orally | 12 | 1 | 11 | 7 |
| no treatment | orally | 6 | 0 | 6 | 7 |

## Summary and conclusions.

1. The haemagglutination reaction by Columbia SK virus is a result of the adsorption of the virus particles to the virus receptors of sheep erythrocytes.

2. The virus is so firmly fixed, that at 37° C. only a partial elution occurs.

3. The virus receptors of sheep erythrocytes can be destroyed by enzyme of *Vibrio cholerae.*

4. Approximately 40 per cent of the mice could be completely protected against the infection with Columbia SK virus by prophylactic administration of cholera filtrate when both the virus and the filtrate are administered by intraperitoneal route. When different routes were used, no protection was obtained.

5. Filtrates prepared of various strains of *Vibrio cholerae* had a different protective effect.

6. Cholera filtrate does not attack the virus, and approximately one half of the treated and infected animals did not develop immunity, which may indicate that the protective activity of the filtrate is due to a (temporary) destruction of virus receptors of the living cell, as a result of which no adsorption and intracellular multiplication of the virus occurs. The virus could be demonstrated 3 days after the intraperitoneal infection in the abdominal cavity of the animals treated with cholera filtrate.

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# INVESTIGATION OF A CASE OF FERMENTATION IN FRUIT PRODUCTS, RICH IN SUGARS

by

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(Received January 29, 1951).

## INTRODUCTION.

Recently two samples of sugared (candied) fruits were sent to our laboratory. The manufacturer had noticed that gas formation had occurred in batches of these fruits. Since he had previously felt that these products were safe for fermentation, as they contained at least 70 % of solids, he asked us for an investigation.

On arrival in the laboratory the fruits present in both samples were cut into pieces of size about 1 cm, and then homogenized with the adherent sugar solution in a stirrer of the Waring-Blendor type which had previously been heated for 1 hour at 80° C. A determination of the refractive index of the syrups, obtained in this way, revealed that they contained abt. 70 % soluble solids. When the syrups were incubated in Einhorn-tubes for 3 days at 30° C. "complete fermentation", i.e. formation of sufficient carbon dioxide to fill the closed part of the tube, occurred.

These observations indicated that the fermentation under consideration was caused by what are called osmophilic yeasts. As only a few cases of such fermentations have been met with in the Netherlands (8), it was decided to study in more detail the cause of the infection and the properties of the microorganisms concerned.

Since a complete review of the cases of osmophilic spoilage, recorded in the literature, has been given recently (12), there is no need to treat this subject in detail again. It may be sufficiently summarized, by saying that in commodities rich in sugars, e.g.

honey, chocolate fillings (1, 13), fondant, sugared fruits, and recently also in citrus concentrates (4, 5) and salad dressings (17, 3), fermentation occasionally has been noticed, which — because the osmotic pressure in the products was at least equal to that of a saturated sucrose solution — could not have been caused by ordinary *Saccharomyces* or *Torulopsis* species. The most important group of organisms, active in these cases, are strains of *Zygosaccharomyces* which are able to ferment sugar solutions up to saturation.

It is regrettable that many of the older reports on this subject have only limited value, since neither the exact composition of the sugar mixture fermented, nor its precise concentration are recorded in many instances. Because the composition at a given total sugar concentration governs the osmotic pressure of the system (9), in this investigation the substrata were fully analyzed.

## METHODS.

### Chemical.

The syrups prepared from the commodities were analyzed for total solids, sugar composition and equilibrium relative humidity.

Total solids were determined directly by a modification of the classic method of JOSSE (6), published recently (10). About 2 ml of the syrup was diluted to about 20 % of total solids (estimated from $n_D^{20}$), adjusted to pH ~ 6.5, and dried at 100° C. after dispersion on a construction made of folded filter paper (10). The time, required for drying was $\leqslant 3$ hours.

The sugar composition of the syrups was estimated according to the scheme developed in the Netherlands by SCHOORL, KOLTHOFF and KRUISHEER. The concentrations of invert sugar and dextrose are calculated from the direct reduction of the LUFF-SCHOORL copper reagent (15) and the reduction resulting when the dextrose present is oxidized by alkaline hypoiodite solution. Sucrose is determined by measuring the increase in the reduction, occurring on inversion at 70° C. following CLERGET (2). Finally, the dextrin content of the systems is determined from the increase in reduction noticed after complete hydrolysis (7).

Equilibrium relative humidities at 25° C. were determined by exposing thin layers ($\leqslant 2$ mm) of the syrups, weighed in in tared weighing vessels, to saturated solutions of the following salts (table I).

Table I.

Saturated salt solutions used for the determination of equilibrium relative humidity (h) at about 25° C.

| h | Salt used | Sol. (25° C.) g/100 ml |
|---|---|---|
| 0.90 | $Na_2CO_3 . 10\ H_2O$ | 29 |
| 0.85 | KCl | 36 |
| 0.80 | $(NH_4)_2SO_4$ | 77 |
| 0.75 | NaCl | 36 |
| 0.70 | KI | 147 |

Equilibration was carried out in desiccators. Since it appeared as a rule, that an equilibrium was not reached within 3—4 weeks [which was to be expected on theoretical grounds (11)] it was necessary to preserve the samples by addition of 0.2 % of benzoic acid.

Before the actual determinations were carried out, samples of the materials were tentatively exposed to humidity standards of the expected order of magnitude. From the weight changes noticed in these experiments two suitable standards were elected, i.e. one showing the lowest relative humidity above that of the sample under investigation and a second, possessing the highest value below that of the sample.

From the weight changes noticed in exposing the samples to these standards, the equilibrium relative humidity was calculated by linear interpolation.

### Microbiological.

The yeasts causing the fermentations were isolated in the following way. The homogenized systems were incubated in Einhorn-tubes at 30° C. until fermentation was "complete", i.e. until enough carbon dioxide was formed to fill the closed part of the tubes. The fermented substrate was then diluted 1 : 3 (vol.) with sterile water and centrifuged for 10 min. at 2500 r.p.m. The sediment was cultured directly on 60 wt % levulose-agar-medium (vide infra).

For isolation and purification of the strains a medium containing about 40 wt % dextrose was used initially. In the course of the investigation it turned out that for samples which showed a mixed flora of osmophilic and ordinary strains, a more selective medium, i.e. one with 60 wt % of a hexose was necessary. Since the solubility of dextrose in aqueous media at room temperature is only of the order of 80 g/100 ml [= 45 wt %[1])], levulose had to be used (solubility of the order of 80 wt %). The medium finally adopted was of the following composition:

Table II.

Medium used in isolating osmophilic strains.

| Component | Quantity |
|---|---|
| Levulose | 600 g |
| Yeast extract, Difco | 5 g |
| Agar, Difco | 25 g |
| Tap water | 370 ml |

Sterilization: 30 min. at 100° C.

The physiological behaviour of the pure isolates was studied by inoculating 40 ml of solutions containing 50, 55, 60, 65 and 70 wt % of levulose (+ 0.5 % of yeast extract Difco) with 0.5 ml of 48—60 hr old cultures of the strains in 50 wt %[1]) dextrose-yeast extract solution. The latter was used as stock solution for reasons of economy.

Proceeding in this way, standard inoculations of about $10^6$ cells/ml were obtained. The inoculated media were incubated in Einhorn tubes at 30° C., and the formation of gas was measured daily.

Identification of the strains was carried out by the Centraal Bureau voor Schimmelcultures, Yeast Division, at Delft. The author is indebted to Mrs N. J. W. Kreger-van Rij for undertaking this investigation.

## COMPOSITION AND INFECTION OF THE SUGARED FRUITS.

The composition of the homogenized mixtures of fruit and adherent sugar solution is shown in Table III.

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[1]) In practice crystallization of sugar starts at concentrations of about 50 wt%. It seemed to be more likely that this is due to supersaturation than to mutarotation phenomena, leading to the better soluble $\beta$-glucose.

Table III.

Properties of the sugared fruits.

| Fruit basis | Physical properties | | | Composition (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| | $n_D^{20}$ | pH | eq.hum. (25° C.) | Tot. solids | Dextrose | Invert sugar | Sucrose | Dextrin |
| Pears | 1.4639 | 4.2 | 0.82 | 69.2 | 16 | 7 | 31 | 16 |
| Cherries | 1.4650 | 3.7 | 0.81 | 69.8 | 15 | $9\frac{1}{2}$ | 31 | 15 |

It can be concluded from these data, that no significant difference in composition existed between the two types of sugared fruits.

The strains, isolated from both commodities, were almost identical as may be illustrated by Table IV.

Table IV.

Physiological properties of the osmophilic strains.

| Strain nr | Visible growth after (days) on levulose agar of | | Complete fermentation after (days) of levulose solutions cont. (wt%) | | | | |
|---|---|---|---|---|---|---|---|
| | 60 wt % | 65 wt % | 50 | 55 | 60 | 65 | 70 |
| I | 4 | 9 | $2\frac{1}{2}$ | $3\frac{1}{2}$ | $3\frac{1}{2}$ | $4\frac{1}{2}$ | 25 |
| II | 5 | 9 | $2\frac{1}{2}$ | 4 | 4 | $4\frac{1}{2}$ | 25 |

Neither one of the strains fermented sucrose [cf. (5)] although they showed definite growth in 30 wt % sucrose-yeast extract broth of pH = 6.2, which had been sterilized by filtration. They were identified as *Saccharomyces Rouxii* Boutroux.

Counts on 60 wt % levulose agar at 30° C. revealed that the fruits contained $10^2$—$10^4$/g of osmophilic yeasts.

## EPIDEMIOLOGY.

The equilibrium relative humidity of the commodities studied is quite consistent with the development of osmophilic yeasts, since the latter are more or less defined as at least capable of developing in saturated sucrose solutions ($h = 0.85$). Some strains indeed seem to thrive at humidities as low as 0.62 (14). Hence any infection with osmophilic strains will result in fermentation of these products. Therefore it was relevant to study the way in which the yeasts had infected the commodities in the present case.

Investigation of this matter showed that at least two sources were to be reckoned with. The first was a batch of beet molasses [already recognized as a possible habitat of osmophilic yeasts before (16)], a sample of which had pH = 4.4 and h = 0.76; it contained 71.5 % solids. The strain isolated from this syrup showed the same physiological properties as the strains nr I and II and was correspondingly identified as *S. Rouxii*.

Further it turned out that the bees, flying on the premises of the factory involved, were carriers of osmophilic yeasts. This evidence was obtained in the following way. Bees were allowed to crawl for about 1 hr on 50 wt % dextrose agar, which then was incubated at 30° C. The yeasts growing on these plates were subcultured on 60 wt % levulose agar and in this way a strain IV was isolated, which again appeared to be *S. Rouxii*.

This finding confirms anew the conception [LOCHHEAD 1929; cf. (12)], that bees are responsible for the contamination of concentrated saccharine foods with osmophilic yeasts.

## Summary.

1. A case of fermentation in sugared fruits (equilibrium relative humidity about 0.82) was studied.

2. The causative yeast was *Saccharomyces Rouxii*. Concentrations up to 70 wt % of levulose in 0.5 % yeast extract solution did not prevent its fermenting. The strains showed visible growth on 65 wt % levulose-yeast extract agar and grew weakly and without fermentation in 30 wt % sucrose-yeast extract solutions.

3. The yeast was also isolated from beet molasses, and from bees found in the factory where the fruit was made.

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# ON THE RELATION BETWEEN TOXIN PRODUCTION AND PROTEIN SYNTHESIS BY *CORYNEBACTERIUM DIPHTHERIAE* AND THE IRON CONTENT OF THE CULTURE MEDIUM

by

**A. TASMAN** and **J. D. VAN RAMSHORST**

(Received January 4, 1951).

In a recent paper Brandwijk, Tasman and van Ramshorst (1) have pointed to the fact that for the purification of diphtheria toxoid it is essential that the ratio of non specific protein/specific antigen in the raw material is as low as possible. This involves in practice the production of diphtheria toxin which contains next to the toxin s.s. a minimum of non specific proteins. *C. diphtheriae* synthesizes during its development the latter proteins. The ratio of specific to non specific proteins depends on various factors among which may be cited: the strain used, the culture medium and the incubation time. In the following we shall make clear that also the iron content of the medium plays an important rôle.

In the paper cited it has been shown that during the development of the culture the production of specific protein (toxin) does not keep pace with that of non specific proteins, the formation of the former initially occurring at a greater rate. So it is profitable to harvest the culture at an earlier date than this is normally done. The optimal incubation time was determined at 5—6 days. In these experiments the ,,Pope medium'' (12) was used as this was normally used in the National Institute for Public Health. The strain we used was a sub-culture of the well-known "P.W.—8" strain (6), obtained from the Wellcome Research Laboratories at Beckenham, labelled "C.N. 2000".

The importance of iron in the production of diphtheria toxin has since long been recognised. Pope (11) is among the first who have shown the connection between toxin production and iron content of the medium. He found a maximal yield of toxin at about 0.5 $\gamma$ Fe per ml broth. His culture medium, however, proved to be rather iron-tolerant, a variation of the iron content between fairly wide limits (0—0.8 $\gamma$ per ml) not perceptibly influencing the yield of toxin.

Pappenheimer and Johnson (10) have investigated this question more thoroughly, using the medium of Wadsworth and Wheeler (13), the main constituent of which is Difco-proteose-peptone. This medium appeared to be actually more "iron-sensitive", so that the latter investigators found a fairly sharp iron optimum at about 0.16 $\gamma$ Fe per ml. Also in his later investigations, where a gelatine hydrolysate medium was applied, Pappenheimer (7) found a same optimal concentration for this element. In 1937 Hettsche (2) published a paper bearing on this subject in which, however, no fresh aspects were presented. Norlin in his thesis (5) reported that an important part of the iron added was taken up by the bacteria. In 1947 an investigation of Pappenheimer (8) appeared in which the question was taken up anew and a quantitative relationship was established between the following three values: iron concentration of the medium, toxin and porphyrin production. He arrived at the following conclusion: for every 4 atoms of iron which occurred in the medium over and above the optimum for the toxin production, 4 molecules of porphyrin and 1 molecule of toxin are complex bound. This "bound toxin" can no longer be detected.

The most probable interpretation of these facts would be in diphtheria toxin being the "protein moiety" of an iron containing respiration enzyme. According to Pappenheimer and Hendee (9) this respiration enzyme would be identical with cytochrome-b.

Finally a paper of Holt (3) may be cited, who also for his medium (casein hydrolysate) established a fairly sharp optimal iron concentration, which was determined for each fresh batch of broth.

All these investigations have been chiefly influenced by the wish to obtain maximal toxin yields (Lf/ml), whilst the "quality" of the toxin is not considered. As is well known this quality is expressed by the number of flocculation units per mg protein nitrogen (Lf/mg P.N.).

In view of the results of Brandwijk, Tasman and van Ramshorst, which clearly show that the "quality" of the toxin depends a.o. on the incubation time of the culture, it seemed to us of interest to ascertain, whether the iron concentration might act as well upon the quality of the toxin produced.

More recently in the National Institute for Public Health an American culture medium, described by LEVINE, WYMAN and EDSALL (4) is routinely used. Its main constituent is a casein hydrolysate (casaminoacids, Difco). This medium induces a lesser synthesis of non specific proteins, compared with the specific toxin proteins. It has been recognised already (1), that this is of great significance for the subsequent purification of the toxoid, prepared from the toxin. As strain a "Toronto" culture was used, obtained from the above investigators.

A batch of 25 l of this medium, its pH after sterilization amounting to 7.50 was prepared. It contained 2.31 % maltose. This volume was divided into 14 lots, to which various amounts of iron as an aqueous solution of 0.01 % $FeSO_4$ 7 aq. were added. Each lot consisted of 6 culture flasks. All flasks were inoculated from one culture and incubated during 6 days. Then the contents of the flasks of a same lot were mixed and filtered.

The following values were determined in these filtrates: Lf/ml, protein nitrogen, Lf/mg P.N., pH, content of maltose and dry weight of bacteria, the latter calculated per l culture medium. The results of this experiment are given in Table I.

Table I.

Relation between toxin production, protein synthesis and the iron content of the medium.

| No. | $\gamma$ Fe per ml | Lf/ml | % P.N. | Lf/mg P.N. | pH | % maltose | gram bacteria per l |
|---|---|---|---|---|---|---|---|
| 1 | 0.01 | 54 | 0.0058 | 930 | 6.55 | 1.75 | 2.68 |
| 2 | 0.04 | 66 | 0.0061 | 1080 | 6.23 | 1.60 | 3.45 |
| 3 | 0.07 | 88 | 0.0102 | 860 | 6.34 | 1.50 | 4.36 |
| 4 | 0.10 | 84 | 0.0116 | 730 | 6.50 | 1.45 | 4.86 |
| 5 | 0.13 | 78 | 0.0110 | 710 | 6.46 | 1.41 | 4.85 |
| 6 | 0.16 | 60 | 0.0113 | 530 | 6.52 | 1.38 | 4.78 |
| 7 | 0.20 | 54 | 0.0117 | 460 | 6.65 | 1.26 | 5.07 |
| 8 | 0.25 | 41 | 0.0099 | 415 | 6.68 | 1.33 | 6.28 |
| 9 | 0.30 | 41 | 0.0100 | 410 | 6.66 | 1.30 | 6.53 |
| 10 | 0.35 | 34 | 0.0109 | 315 | 6.63 | 1.35 | 5.65 |
| 11 | 0.40 | 32 | 0.0095 | 335 | 6.67 | 1.34 | 5.61 |
| 12 | 0.50 | 20 | 0.0105 | 190 | 6.67 | 1.31 | 5.77 |
| 13 | 0.60 | 10 | 0.0110 | 90 | 6.72 | 1.31 | 5.86 |
| 14 | 0.70 | 3 | 0.0099 | 30 | 6.67 | 1.29 | 5.73 |

It appears that pH and the content of maltose vary but slightly, depending on the iron content of the medium. The most important is the slope of the "toxin"- and the "purity curve", designated as Lf/ml and Lf/mg P.N. The maximum of the "purity curve" appears to occur at a lower iron concentration than for the "toxin curve" (see also fig. 1).

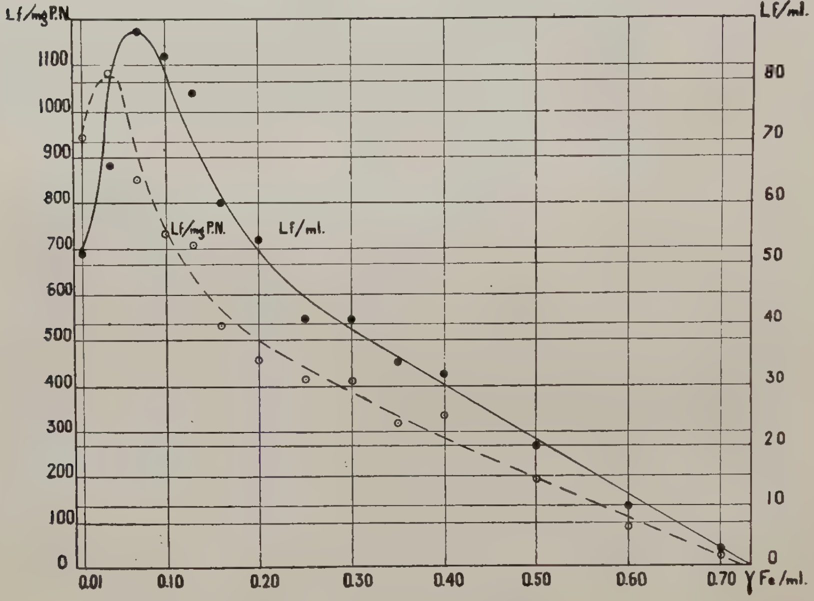

Fig. 1.

We will not go into an explanation of these facts. The nature of the toxin production and of the synthesis of non specific protein by *C. diphtheriae* is still so obscure that a speculation about the facts reported seems to us to be of slight value.

The consequences of the correlation recognised are, however, of practical importance. We have repeated these experiments several times with essentially the same results. An example is given in Table II.

The results pointed always in the same direction. The optimal iron concentration for the "purity" of the toxin obtained does not coincide with that for the maximal yield of toxin, but is always lower than the latter.

Brandwijk, Tasman and van Ramshorst have already reported that the "purification" of the diphtheria toxoid used in the modern

Table II.

Relation between toxin production, protein synthesis and the iron content of the medium.

| No. | $\gamma$ Fe per ml | Lf/ml | % P.N. | Lf/mg P.N. |
|---|---|---|---|---|
| 1 | 0.01 | 50 | 0.0055 | 910 |
| 2 | 0.08 | 71 | 0.0099 | 720 |
| 3 | 0.15 | 68 | 0.0100 | 620 |
| 4 | 0.21 | 57 | 0.0117 | 490 |
| 5 | 0.34 | 26 | 0.0131 | 200 |
| 6 | 0.71 | $<8$ | — | — |

vaccines, presents great difficulty. This is chiefly owing to the fact that specific and non specific protein formed by *C. diphtheriae* during its growth differ but very slightly physico-chemically. So it stands to reason that for the ultimate purification it is of utmost importance that the initial material is as pure as possible. The final conclusion drawn in our previous paper remains valid, *viz.*, that the preparation of a diphtheria toxin as pure as possible (Lf/mg P.N.) has to be aimed at, and that for that sake a lower yield of toxin (Lf/ml) than might be obtained with an optimal iron concentration, has to be accepted.

## Summary.

An investigation of the quantitative relationship between toxin production and protein synthesis by *C. diphtheriae* and the iron content of the medium has been reported on. Both functions can be expressed quantitatively, *viz.*, Lf/ml and Lf/mg P.N.

Both functions appeared to be narrowly related to the iron concentration of the medium. The optimal Fe-concentration for the greatest purity of the toxin (Lf/mg P.N.) lies, however, lower than that for the maximal yield of toxin (Lf/ml).

In connection with the fact that for the subsequent purification of the toxoid to be obtained from the diphtheria toxin, starting from as "pure" as possible raw material is essential, a somewhat lower production of toxin than an optimal iron concentration might yield, has to be accepted.

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# THE ADDITION OF TRISODIUMPHOSPHATE SOLUTION TO GASTRIC LAVAGES FOR THE CULTIVATION OF *MYCOBACTERIUM TUBERCULOSIS*

by

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(Received January 24, 1951).

The culturing of gastric lavages for tubercle bacteria, which is at present a valuable diagnostic method in clinics and dispensaries, cannot be applied in central public health laboratories to the extent that the importance of the method deserves (BEKKER (3)). Because of the great distances to laboratories the gastric lavages are generally eliminated, while the notorious deleteriousness of the gastric juice for the tubercle bacteria deprives a negative result of any meaning and the culturing test of reliability.

To overcome this difficulty ARMSTRONG (1) recommended the addition of an equal part of 10 % $Na_3PO_4$ solution (or 23 % $Na_3PO_4$ 12 aq.) to the gastric lavages, and preliminary treatment according to CORPER and STONER, in the laboratory. CORPER and STONER (4) had been the first to suggest "digestion" in the incubator at 37° C. (of sputa and other materials) with the $Na_3PO_4$ solution for one, or by convenience for two and more days, in culturing for tubercle bacteria. In this way they claim to have achieved better results than by homogenising with 3 % NaOH or 5 % oxalic acid. Before the inoculation it was necessary only to concentrate the homogeneous mixture (20 mins. centrifuge at 2500 r.) and neutralize the concentrate. The essential virtue of the procedure is that the high alkalinity of the trisodiumphosphate solution should destroy the contaminating organisms completely, while it should not be lethal to tubercle bacteria even in eight days — at least at room temperature.

VAN VRANKEN (12) employed the procedure, seeing the simplification as a benefit for public health laboratories. It could compete with the oxalic acid preparation: 127 positive cultures against 108 with the latter, in 1000 samples of sputa and other materials. Armstrong emphasized some shortening of the time on the average needed for visibility of the culture, and the improvement of the quality of the concentrates. Besides this his good opinion was based on the inconclusive fact that the incidence of cases in which a positive direct smear of concentrate had been followed by a negative culture, had been reduced "nearly to zero".

The experiments recorded here, which lack moreover a comparison with the usual sulfuric acid preliminary treatment of Loewenstein-Simuyoshi, could not entice to disloyalty to the latter well-tried, brief method, but could excite an endeavour to investigate empirically the utility of the $Na_3PO_4$ solution to gastric lavages. This paper is a report on the results of that investigation.

A few preliminary notes must be made. It is not as yet known by what mechanism $Na_3PO_4$ checks the deleteriousness of the gastric juice for the tubercle bacteria in spite of its doubtlessly detrimental high alkalinity. The deleteriousness which is such that the culturing of positive gastric lavages fails within 24 hours to furnish a culture of *M. tuberculosis* (Schwarting (8), Floyd and Page (5), Sprick and Towey (10), Vincent and Birge (11) a.o.), has been imputed to the hydrochloric acid, scil. the high hydrogen ion concentration of the gastric juice. However, the neutralization of the small quantities of HCl by the $Na_3PO_4$ must be of secondary importance. There is evidence that much greater concentrations of hydrochloric acid than those of the gastric juice are not so noxious as the gastric juice itself (Roper and Ordway (7), Kramer (6)). So the digestive enzymes are today thought to be the chief factor in destroying the tubercle bacteria. This explains easily the retarding effect of refrigeration and of neutralization.

The investigation was carried out by comparing the results of the cultivation on Loewenstein media, after the preparation according to Corper and Stoner, with those after the direct treatment of the fresh material with 3 % hydrochloric acid, which is the usual practice in this laboratory ¹), both methods on the same

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¹) Procedure: Concentrate by centrifuge, mix with about 4 parts of HCl (conc. 30 ml aq. ad 1000 ml), shaking apparatus 15 mins, standing 30 mins, concentrate and wash with sterile 0.85 % NaCl. This procedure is, according to Bekker (not published), slightly superior to the treatment with 6 % sulfuric acid, as far as gastric washings are concerned.

gastric lavages. Dr H. C. Hallo cooperated in splitting carefully the gastric lavages of patients of the Hilversum dispensary in two equivalent parts, placing one into a jar containing the $Na_3PO_4$ solution, the other into an empty one. The jars reached the laboratory within an hour. The first jar remained one day at room temperature and two days in the incubator at 37° C., while the latter one was treated immediately in the usual way. Table 1 (column *a*) shows the results.

Table 1.

| | | *a.*<br>1 day room temperature and 2 days 37°C. in 5% $Na_3PO_4$ solution | | | *b.*<br>1 day room temperature in 5% $Na_3PO_4$ solution | | |
|---|---|---|---|---|---|---|---|
| acid treatment | $Na_3PO_4$ treatment | number | % | standard deviation | number | % | standard deviation |
| + | + | 12 | 5.7 | 1.5 | 73 | 12.3 | 1.1 |
| + | — | 27 | 11.8 | 1.2 | 35 | 5.9 | 0.5 |
| — | + | 1 | 0.5 | 0.3 | 30 | 5.0 | 0.4 |
| — | — | 188 | | | 457 | | |
| ratio $\frac{Na_3PO_4 \text{treatment}}{\text{acid treatment}}$ | | | 0.31 | (0.07) | | 0.95 | (0.04) |

The treatment according to Corper and Stoner for two days appears much less efficacious than the HCl-treatment: it failed to detect 27, or 67 % of the positives. The difference of 6.2 % positive cultures against 17.5 % by the usual method is significant, and reveals the damaging of *M. tuberculosis* by $Na_3PO_4$, a fact that is not astonishing when the high alkalinity of the material is considered. Nevertheless the alleged saving effect of the $Na_3PO_4$ does indeed exist, though it results, in the procedure of Corper and Stoner, only in a comparative improvement in relation to gastric lavages that have stood a few days without $Na_3PO_4$ solution.

The next step in the project was the examination of the gastric lavages in the same way, except for the "digestion" in the incubator 37° C. This temperature undoubtedly increases the damage the $Na_3PO_4$ does to the tubercle bacteria; moreover it appeared that this temperature was not needed to make the mixture homogeneous. The killing of the contaminants is according to Corper and Stoner efficient within 24 hours at room temperature. The results were almost surprising (column *b*, table 1): 17.3 % positive cultures. This percentage is within the limits of probability equal to the percentage of positive cultures resulting from the HCl treatment. The difference to the 6.2 % of the digestion at 37° C. is significant. Indeed the latter seems very harmful. But, above all, the utility of adding 10 % $Na_3PO_4$ solution to gastric washings in culturing for tubercle bacteria is evident, provided the temperature of the mixture remains lower than about 20°C.

The figures of table 1 do not prove with certainty the approximate equivalence (ratio 0.95 ± 0.04) of the $Na_3PO_4$ treatment at room temperature and the acid treatment, while the splitting of a gastric lavage into two exactly equivalent parts is a matter of chance. One part may contain in a flock of mucus all of the cultivable organisms. Accordingly, a great number (65) of the positive cultures has been obtained by only one of both methods. So a chance unequal distribution of the half-parts with tubercle bacteria into the two kinds of jar may have flattered the number of positives after the $Na_3PO_4$ treatment, as well as after the acid treatment. The probability of the distribution of half of the 65 parts with tubercle bacteria into one kind of jar (50 %) has a standard deviation of 6.2 %. Based on this, we calculated 1.04 and 0.82 as the most probable maximum-minimum values of the ratio $Na_3PO_4$-positives (at room temperature): acid-positives; and 1.21 and 0.71 as the limits of the range of possible values by chance. This means that the figures of column *b* do not exclude a superiority of the $Na_3PO_4$ treatment over the usual acid treatment up to 21 %.

The last step in the evaluation of the addition of $Na_3PO_4$ solution to gastric lavages was the examination of equivalent material with both methods. The equivalence was checked by microscopic enumeration of the bacteria in smears. The figures of table 2 reveal that even at room temperature $Na_3PO_4$ reduces the number of cultivable tubercle bacteria materially. This corresponds, we believe, to the slight inferiority of the $Na_3PO_4$ treatment at room temperature, which we ascertained as most probable by comparison with the usual acid treatment.

Table 2.

| | | Number of colonies of tubercle bacteria. |
|---|---|---|
| after acid preliminary treatment | | 100 |
| after $Na_3PO_4$ treatment | 24 hours room temperature | 39 |
| after ,, ,, | 24 hours at 37° C. | 4.8 |
| after ,, ,, | 3 to 4 hours at 37° C. | 10—70 |

The last two lines of table 2 bear out the conclusion concerning the deleteriousness of the $Na_3PO_4$ solution at 37° C.

The more rapid growth of the culture after the preliminary treatment with $Na_3PO_4$, which ARMSTRONG mentioned, was also evident in our investigation: 22 % of the positives by both methods could be obtained one week or more earlier after the $Na_3PO_4$ treatment. There was not much in the other recorded advantages. The killing of the contaminants was as efficient as that achieved by hydrochloric acid: there was no significant difference in the percentage of contaminated cultures ($< 1$ %).

The papers which have been published in the course of this investigation give a similar view of the influence of $Na_3PO_4$ on *Mycobacterium tuberculosis.* BEATTIE (2) did not find a significant difference between the hydrochloric acid treatment of sputa and the $Na_3PO_4$ treatment. SPENDLOVE *et al.* (9) showed that 5 % $Na_3PO_4$ solution (to 3 days 37° C.) is the least harmful of the many sputum-digestants, in the long run, but half of the tubercle bacteria in the 5 % $Na_3PO_4$ solution were not cultivable after 40 minutes and later.

## Conclusions.

The addition of a 10 % trisodiumphosphate solution to fresh gastric lavages is applicable for the cultivation of *M. tuberculosis* from gastric lavages in a laboratory at a great distance. However, the material has to be kept as cool as possible in order to achieve results that rank only slightly below those achieved by immediate preliminary treatment with 3 % hydrochloric acid, at least when the examination takes place within 24 hours.

The addition of the 10 % trisodiumphosphate solution to other materials than gastric lavages (sputa, etc.) is not motivated by any sound principle, and is objectionable because of the noxious action of the $Na_3PO_4$ on the tubercle bacteria, especially at summer temperature.

## Summary.

The utility of the addition of an equal amount of a 10 % $Na_3PO_4$ solution to gastric lavages in culturing for *Mycobacterium tuberculosis* has been investigated and has been found applicable under certain conditions, on the understanding that before and during transport the mixture is kept at temperatures not exceeding 20° C.

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# SOME OBSERVATIONS ON ACQUIRED RESISTANCE OF TUBERCLE BACTERIA TO 4-ACETYLAMINOBENZALDEHYDE THIOSEMICARBAZONE

by

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In the treatment of tuberculosis in man three substances have at present gained considerable interest: streptomycin, para-aminosalicylic acid (P.A.S.) and 4-acetylaminobenzaldehyde thiosemicarbazone (TbI, Conteben). The application of these agents, in combination or alone has been generally recognized as an important approach in the control of this disease.

One of the most severe limitations in the use of streptomycin and P.A.S. is the tendency of tubercle bacteria to become drug-resistant (3, 10, 12). The rate at which this effect arises varies with respect to substance and strain used. It was found to be in general much more difficult to get bacteria by in vitro experiments resistant to P.A.S. than to streptomycin (3). In the case of P.A.S. acquired resistance of tubercle bacteria to this material has even been denied (cf. (3)).

Drug-resistant tubercle bacteria do not seem to be detected yet in treatment with the most recent chemotherapeutic agent 4-acetylaminobenzaldehyde thiosemicarbazone (Mertens and Bunge (9), Domagk (4)). This property has been considered a material advantage of the drug over streptomycin and P.A.S. (9). However, for two reasons this should not be taken too positive. Firstly, because hitherto the substance under consideration has mainly received clinical interest, whereas investigations of its properties in the laboratory still seem scanty; in the early stage of clinical and laboratory experience with P.A.S. the same erroneously being held for this material. Secondly, because of the fact that in vitro development of resistance in tubercle bacteria has recently been reported in the case of a chemically related substance (11).

These considerations prompted us to investigate whether in vitro tubercle bacteria could be adapted to the inhibitory action of thiosemicarbazone.

Up till now our observations bear upon three strains, two belonging to the human type (strain 18a and 46861), and one a strain of *Mycobacterium avium*. Strain 18a had been previously isolated from an infected cavia. Strain 46861 was isolated from a patient which had been treated for several months with P.A.S. This strain was found to be highly resistant to the drug, even the presence of 1000 $\mu$g of P.A.S. per ml of the medium not being able to stop growth. The avian strain was received through the courtesy of Dr G. M. van Waveren, Director of the State Serological Institute at Rotterdam and had previously never been in contact with drugs. Investigations on more strains, including one of the bovine type, have been started now.

The medium used in the investigation was Herrolds. This medium was prepared as follows. A solution of 0.8 per cent nutrient broth (Difco) and 1.5 per cent glycerol was prepared in tapwater. The pH of the solution was adjusted to 7.5 using 0.1 N NaOH, and 2 per cent acetone-extracted bacto-agar (Difco) was added¹). After autoclaving for one hour at 115° C. and subsequent cooling down to 60°C. one egg yolk was added to each 150 ml portion of melted agar. Thiosemicarbazone may be added to this medium by dissolving the substance in propyleneglycol and making serial dilutions in agar. Concentrations higher than 50 $\mu$g/ml were prepared from sterile suspensions of the drug in bouillon. The thiosemicarbazone solution in propyleneglycol has to be made bacteria-free by filtering through a Chamberland candle $L_3$. The final concentration of the solvent may not surpass 0.5 per cent. Throughout the investigation slants containing 0 — 0.1 — 0.2 — 0.5

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¹) Commercial preparations of agar may exert a pronounced inhibitory effect on the growth of tubercle bacteria (Dubos and Middlebrook (5), Beeuwkes (1)) most probably due to the presence of long chain fatty acids (5). According to Beeuwkes (1) a 24 hours acetonic extraction of the agar prior to its use may result in a better proliferation. The correctness of this observation could be confirmed in our experiments.

— 1 — 2 — 5 — 10 — 20 — 50 — 100 — 200 and 500 μg/ml of thiosemicarbazone were used. Higher concentrations were not included in the investigation since an amount of 500 μg/ml is already far above the solubility of the substance in water, this being 172 μg/ml at 37° C. (2).

The technique employed in order to produce resistant strains was exposing the bacteria to gradually increasing sub-inhibitory concentrations of the drug. This procedure was continued until all strains had been adapted to high concentrations. The results obtained are shown in the table.

As may be seen from the table resistance to thiosemicarbazone in the bacteria emerges predominately after the cultures have been exposed to the drug for two months. Fully resistant strains are obtained after about four months of exposure. An amount of even 500 μg/ml is not able to induce a complete suppression of growth; the drug, however, retards proliferation considerably.

In the early stage of acquiring resistance to thiosemicarbazone the development of rapidly proliferating single colonies occurs in both the human strains. This is highly reminiscent of what has been seen in the in vitro development of resistance to streptomycin. However, the impression is gained that resistance to streptomycin arises more quickly. In some experiments with P.A.S. running parallel with those of thiosemicarbazone some evidence was gained that in the case of *Mycobacterium avium* resistance to the former substance is somewhat slower to arise. This was evident as the time required to get a strain resistant to 500 μg/ml of thiosemicarbazone was about the same as the time to adapt the bacteria to the presence in the medium of 100 μg/ml P.A.S. In the case of strain 18a, on the other hand, not the slightest increase of resistance to P.A.S. could be noted.

Finally, attention may be directed to the interesting feature that thiosemicarbazone may often show a concentration range where growth inhibition is less than at adjacent regions of higher or lower concentrations (see table). This phenomenon has perhaps been observed more clearly in several dithiocarbamic acid derivatives (6), which possess a notorious toxicity to moulds as well as to some bacteria including mycobacteria (7, 8). As, inversely, also thiosemicarbazones have been reported to possess antifungal properties (4), the nature of the characteristic effects of the substances under consideration upon moulds and bacteria must

Table showing acquisition of resistance to 4-acetylaminobenzaldehy

| | | Concentration in μg/ml | *Mycobacterium avium* | | |
|---|---|---|---|---|---|
| | | | readings taken after: | | |
| | | | 12 days | 21 days | 31 days |
| Growth of parent strains | | 0 | ++ | +++ | +++ |
| | | 0.1 | ++ | +++ | +++ |
| | | 0.2 | + | ++ | +++ |
| | | 0.5 | ++ | +++ | +++ |
| | | 1 | — | ++ | +++ |
| | | 2 | — | + | + |
| | | 5 | — | — | — |
| | | 10 | — | — | — |
| | | 20 | — | — | — |
| | | 50 | — | — | — |
| | | 100 | — | — | — |
| | | | 10 days | 16 days | 31 days |
| Growth of substrains I | subcultered from parent strains after 31 days growth at 1 μg/ml of the drug | 0 | ++ | +++ | +++ |
| | | 0.1 | ++ | +++ | +++ |
| | | 0.2 | + | +++ | +++ |
| | | 0,5 | ++ | ++ | +++ |
| | | 1 | — | + | +++ |
| | | 2 | — | — | ++ |
| | | 5 | — | — | ++ |
| | | 10 | — | — | ++ |
| | | 20 | — | — | + |
| | | 50 | — | — | — |
| | | 100 | — | — | — |
| | | | 14 days | 22 days | 26 days |
| Growth of substrains II | subcultered from substrains I after 31 days growth at 10 or 20 μg/ml (in the case of strain 46861) of the drug | 0 | +++ | +++ | +++ |
| | | 5 | +++ | +++ | +++ |
| | | 10 | +++ | +++ | +++ |
| | | 15 | +++ | +++ | +++ |
| | | 20 | +++ | +++ | +++ |
| | | 37.5 | +++ | +++ | +++ |
| | | 50 | ++ | +++ | +++ |
| | | 75 | ++ | +++ | +++ |
| | | 100 | + | +++ | +++ |
| | | | 14 days | 21 days | 28 days |
| Growth of substrains III | subcultered from substrains II after 26 days growth at 100 μg/ml of the drug | 0 | ++ | +++ | +++ |
| | | 50 | ++ | +++ | +++ |
| | | 100 | + | ++ | +++ |
| | | 200 | + | + | ++ |
| | | 500 | + | + | ++ |

iosemicarbazone. The plusses refer to the degree of growth.

| Strain 18a |  |  | Strain 46861 |  |  |
|---|---|---|---|---|---|
| readings taken after: |  |  | readings taken after: |  |  |
| 12 days | 21 days | 31 days | 12 days | 21 days | 31 days |
| + + | + + + | + + + | + + | + + + | + + + |
| + | + + + | + + + | + + | + + + | + + + |
| — | — | — | — | — | + + |
| + | + + + | + + + | + + | + + + | + + + |
| — | +¹) | + +¹) | — | +¹) | + +¹) |
| — | +¹) | + +¹) | — | +¹) | + +¹) |
| — | — | — | — | + | + +¹) |
| — | — | — | — | + | + +¹) |
| — |  | — | — |  | + |
| — | — | — | — | — | — |
| — | — | — | — | — |  |
| 10 days | 16 days | 31 days | 10 days | 16 days | 31 days |
| + + | + + | + + + | + + | + + + | + + + |
| — | — | + + + | + + | + + + | + + + |
| + | + | + + + | + + | + + + | + + + |
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| — | — | — | — | — | + + + |
| — | — | — | — | — | + |
|  | — | — | — | — | — |
| 14 days | 22 days | 26 days | 14 days | 22 days | 26 days |
| + + + | + + + | + + + | + + + | + + + | + + + |
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| 14 days | 21 days | 28 days | 14 days | 21 days | 28 days |
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|  |  |  | + | + + | + + + |
| + | + | + + | + | + + | + + + |

) single, rapidly proliferating colonies grow out.

probably been sought in the chemical relation between both classes of substances.

## Summary.

Three strains of tubercle bacteria, two belonging to the human type and one of avian origin, were made resistant to 4-acetylaminobenzaldehyde thiosemicarbazone (Conteben) by being grown in progressively increasing concentrations of the drug using a Herrold agar medium. It could be shown that within four months the strains may be adapted to grow in the presence of 500 μg/ml of thiosemicarbazone, while at the beginning of treatment the bacteria were susceptible to an amount as low as 1 μg/ml of the substance. The impression was gained that resistance to streptomycin may be produced more readily than to thiosemicarbazone, and to the latter substance more easily than to P.A.S.

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# ON THE SENSITIVITY OF THE METHANE FERMENTATION OF CALCIUM ACETATE TOWARDS SOME TOXIC SUBSTANCES

by

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## § 1. Introduction.

In a previous investigation (Noordam-Goedewaagen, Manten and Muller (19) it was found that the methane fermentation of sodium and calcium acetate is quite sensitive towards sulphite, 0.01 % $Na_2SO_3$ giving an inhibition of 29 % of the fermentation of calcium acetate (l.c. fig. 10) and of 50 % of that of sodium acetate at pH 6.0 (l.c. fig. 4). Although sulphite (or sulphur dioxide) is often used as a preservative, it is usually applied in a higher concentration (e.g. 0.1 %).

As sulphite readily combines with aldehyde groups, it was considered possible that its toxic action might consist of a reaction with such groups in the enzyme system of methane fermentation of acetate. Therefore, it was decided to investigate the action of some other aldehyde reagents, *viz.*, hydroxylamine, phenylhydrazine and dimedone (5.5-dimethyl-cyclohexane-dione), upon the fermentation of calcium acetate [1]). For comparison sulphite and some toxic substances of a different character (azide, cyanide, sulphide and formaldehyde) were also included.

Similarly as in the previous investigation purified cultures in 300 ml bottles on a mineral medium with 1 % Ca-acetate and with an asbestos layer were used, the toxic substances being added to the fresh medium replacing the exhausted one over the asbestos layer.

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[1]) Ca-acetate has the advantage over Na-acetate of giving a stronger fermentation and maintaining an approximately constant pH (l.c. pp. 79 and 82) during fermentation.

## § 2. RESULTS OBTAINED WITH THE VARIOUS TOXIC SUBSTANCES, EXCEPT FORMALDEHYDE.

The experiments were made with series of 10 or 6 cultures, including 2 blanks, at a temperature of 30° C. The cultures of a series had been tested for uniformity of fermentation rate beforehand. The fermentation gas was collected over sodium hydroxide solution. The results are given in table I.

Table I.

Inhibition by various substances.

| Cultures | Substance added | Molarity | % | Gas production on consecutive days in ml | | | | | % inhibition |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | |
| E,4 | blanks | — | — | 128 | 155 | 89 | 55 | 49 | — |
| F,L | $Na_2SO_3$ | $10^{-3}$ | 0.0126 | 129 | 159 | 117 | 83 | 53 | 0 |
| G,H | ,, | $10^{-2}$ | 0.126 | 36 | 50 | 42 | 38 | 38 | 70 |
| A,B | blanks | — | — | 125 | 189 | 139 | 79 | — | — |
| C,D | $NH_2OH$ | $10^{-3}$ | 0.0033 | 58 | 103 | 112 | 103 | — | 49 |
| F,H | ,, | $10^{-2}$ | 0.033 | 9 | 11 | 9 | 12 | — | 94 |
| E,K | $C_6H_5N_2H_3$ | $10^{-3}$ | 0.0108 | 75 | 118 | 133 | 125 | — | 39 |
| G,L | ,, | $10^{-2}$ | 0.108 | 23 | 30 | 22 | 38 | — | 83 |
| K,H | blanks | — | — | 179 | 152 | 106 | 81 | 56 | — |
| G,L | dimedone | $10^{-3}$ | 0.014 | 175 | 161 | 109 | 65 | 42 | 0 |
| C,E | ,, | $10^{-2}$ | 0.14 | 131 | 143 | 107 | 71 | 54 | 17 |
| F,D | $NaN_3$ | $10^{-3}$ | 0.0065 | 111 | 118 | 79 | 64 | 58 | 31 |
| A,D | ,, | $10^{-2}$ | 0.065 | 17 | 0 | 0 | 0 | 0 | 95 |
| E,4 | blanks | — | — | 162 | 151 | 79 | 56 | 45 | — |
| C,K | KCN | $10^{-3}$ | 0.0065 | 1 | 0 | 0 | 0 | 0 | 100 |
| B,3 | ,, | $10^{-2}$ | 0.065 | 1 | 0 | 0 | 0 | 0 | 100 |
| G,H | $Na_2S$ | $10^{-3}$ | 0.0078 | 144 | 160 | 126 | 79 | 49 | 1 |
| F,L | ,, | $10^{-2}$ | 0.078 | 166 | 183 | 118 | 74 | 46 | 0 |
| E,4 | blanks | — | — | 174 | 142 | 79 | 57 | 57 | — |
| G,L | KCN | $5.10^{-4}$ | 0.0033 | 19 | 32 | 40 | 37 | 30 | 84 |
| D,F | $Na_2S$ | $5.10^{-2}$ | 0.39 | 59 | 33 | 34 | 34 | — | 71 |
| E,4 | blanks | — | — | 172 | 147 | 121 | 60 | 39 | — |
| C,H | KCN | $5.10^{-4}$ | 0.0033 | 4 | 5 | 20 | 21 | 26 | 97 |
| A,K | $Na_2S$ | $5.10^{-2}$ | 0.39 | 1 | 0 | 2 | 3 | 4 | 100 |

The cultures belonging to each series are grouped together, headed by the respective blanks. Attention is drawn to the fact that most cultures appear in more than one series. At the end of a serial experiment the medium in the bottles was replaced by fresh medium without toxic substances so that the inhibited bacteria could recover; the cultures were not used for a new series before recovery was complete. Thus, this part of the investigation was carried out with merely 12 cultures. The duration of the recovery period varied rather widely and sometimes amounted to a couple of months. As the investigation was carried out along with other work, this did not constitute a drawback.

The concentrations of the toxic substances refer to the replacing medium. Therefore, the concentration in the bottle as a whole will have been somewhat lower (about 5/6 of that listed in table I, the volume of the asbestos layer being about 1/6 of that of the bottle).

The gas production figures are the averages for the two cultures listed in the first column.

The percentage inhibition was calculated from the sum of the gas production of the first two days (the third day already shows a decrease of the fermentation of the blanks, due to exhaustion of the acetate in the medium).

The sulphite produced an inhibition of the same order of magnitude as in the previous investigation (l.c. fig. 10: 0.01 % ± 30, 0.05 % ± 50 and 0.15 % ± 75 % inhibition). Hydroxylamine and phenylhydrazine are somewhat more active than sulphite in a Ca-acetate medium and about as active as sulphite in a Na-acetate medium at pH 6.0 (l.c. fig. 4). However, dimedone was only slightly inhibitory.

Of the other substances azide is about as active as hydroxylamine and hydrazine. At a concentration of $10^{-2}$ m inhibition is complete from the 2nd day onward, whereas with $10^{-2}$ m hydroxylamine fermentation continues at the same low rate. Nevertheless, cultures A and D did recover from this dose of azide, as shown by their use in later series (near the bottom of table I).

Methane fermentation of acetate is remarkably sensitive to cyanide, even $5.10^{-4}$ m causing an almost complete inhibition. Also here recovery from a completely inhibitive dose is possible (cultures C and K were first exposed to $10^{-3}$ m and used later on in the last series of table II).

However, sensitivity to sulphide is quite slight, as $10^{-2}$ m does not inhibit at all. This is not surprising, since methane fermentation and sulphate reduction often occur side by side. In the last series but one $5.10^{-2}$ m gives 71 % inhibition. However, this rather large amount of $Na_2S$ has a distinct influence on the pH of the medium: at the end of the experiment culture D had a pH of 9.1 and F of 7.8. Therefore, the experiment with $5\ 10^{-2}$ m $Na_2S$ was repeated in the last series, with adjustment of the pH of the replacing medium containing $Na_2S$ to 6.3 by the addition of some acetic acid. Now inhibition was complete, while the final pH's were 6.9 for culture A and 7.1 for K. Apparently the sensitivity to sulphide increased with decreasing pH.

## § 3. RESULTS OBTAINED WITH FORMALDEHYDE.

Formaldehyde was included also because, from experiments which cannot be discussed in this paper, the impression was obtained that methane bacteria were capable of fermenting formaldehyde. For this reason the experiments were made in a different way. Two cultures were accustomed to formaldehyde by gradually increasing the concentration in the replacing medium. The behaviour of these cultures appears from table II.

The smallest concentration of formaldehyde employed (0.01 %) already caused some inhibition (the last period without formaldehyde serving as a blank in this experiment). The inhibition increased with the formaldehyde concentration to 80 % for 0.1 % formaldehyde ($3.3.10^{-2}$ m). In the 5 following replacements (from 2/13—3/29 '50) the acetate concentration was gradually reduced to zero, the formaldehyde concentration being maintained at 0.1 %. As the fermentation rate under the conditions employed is quite sensitive to the acetate concentration (l.c. fig. 1), no figures for the % inhibition could be calculated for these and the following periods.

The fermentation rate continued to decrease with the decreasing acetate concentration, but gas production did not stop altogether when no more acetate was added to the replacing medium (3/29 '50 in table II).

Fermentation continued at a rate of 4—5 ml a day till 4/21, when the medium was replaced by one of the same composition. This time the fermentation rate was still lower (2—3 ml/day for a period of 14 days). From 5/6 till 6/22 the cultures produced very little gas and on the latter date some 36 % formaldehyde solution (equivalent to 250 ml 0.1 %, the usual volume of the replacing

Table II.

Action of gradually increased concentrations of formaldehyde.

| Date of replacement of medium | % acetate | % formaldehyde | Total gas production first 2 days in ml | | % inhibition | |
|---|---|---|---|---|---|---|
| | in replacing medium | | I | II | I | II |
| 11/29 '49 | 1.00 | — | 381 | 382 | — | —x |
| 12/5 '49 | 1.00 | 0.01 | 363 | 318 | 5 | 17x |
| 12/12 '49 | 1.00 | 0.02 | 358 | 293 | 6 | 23x |
| 12/19 '49 | 1.00 | 0.03 | 296 | 255 | 22 | 33x |
| 12/28 '49 | 1.00 | 0.04 | 250 | 244 | 34 | 36x |
| 1/2 '50 | 1.00 | 0.05 | 237 | 187 | 38 | 51x |
| 1/9 '50 | 1.00 | 0.06 | 185 | 152 | 51 | 60x |
| 1/16 '50 | 1.00 | 0,07 | 143 | 121 | 62 | 68x |
| 1/23 '50 | 1.00 | 0.08 | 111 | 109 | 71 | 71x |
| 1/30 '50 | 1.00 | 0.09 | 85 | 85 | 78 | 78x |
| 2/6 '50 | 1.00 | 0.10 | 74 | 83 | 81 | 78x |
| 2/13 '50 | 0.50 | 0.10 | 50 | 61 | — | —x |
| 2/22 '50 | 0.25 | 0.10 | 26 | 34 | — | —x |
| 3/6 '50 | 0.10 | 0.10 | 16 | 20 | — | —x |
| 3/17 '50 | 0.05 | 0.10 | 11 | 16 | — | —xx |
| 3/29 '50 | — | 0.10 | 9 | 10 | — | —xx |
| 4/21 '50 | — | 0.10 | 5 | 6 | — | —xx |
| 6/22 '50 | — | 0.10 | 12 | 18 | — | —xx |
| 7/6 '50 | — | 0.10 | 28 | 45 | — | —xx |
| 7/13 '50 | — | 0.15 | 49 | 61 | — | —xx |
| 7/24–21 '50 | — | 0.20 | 18 | 46 | — | —xx |

x Gas collected over NaOH solution.
xx Gas collected over saturated NaCl solution.

medium) was added. Gas production started immediately at a rate of 6 and 9 ml/day resp. and decreased but slowly during a fortnight. This addition of concentrated formaldehyde solution was repeated on 7/6 which resulted in an appreciably higher gas production (12 and 18 ml/ day resp.).

On 7/13 an amount of formaldehyde solution equivalent to 250 ml 0.15 % was added resulting in a still higher gas production (18 and 25 ml/day resp.). On 7/24 and 7/21 resp. this was repeated with an amount equivalent to 250 ml 0.2 % formaldehyde, but this dose appeared too be to high: after a few days gas production stopped and, in the case of culture I, was not resumed when after a week the medium was replaced by one containing the mineral salts + 0.05 % formaldehyde (culture II was not investigated in this respect).

From 3/17 onward the gas was collected over saturated NaCl solution and analyzed at appropriate intervals. The data obtained are collected in table III.

Table III.

Composition of gas produced from formaldehyde.

| Period | Gas collected ml | | Composition % | | | | $CH_4/CO_2$ ratio | |
|---|---|---|---|---|---|---|---|---|
| | | | $CH_4$ | | $CO_2$ | | | |
| | I | II | I | II | I | II | I | II |
| 3/17–29 | 62.7 | 80.2 | 68.6 | 75.6 | 4.6 | 6.6 | 14.9 | 11.5 |
| 3/29–4/14 | 68.8 | 86.0 | 77.9 | 81.5 | 10.2 | 9.4 | 7.6 | 8.7 |
| 4/14–4/21 | 10.8 | 25.0 | — | — | — | — | — | — |
| 4/21–6/22 | 78.2 | 82.0 | 67.9 | 82.7 | 6.4 | 5.4 | 10.6 | 15.3 |
| 6/22–7/6 | 75.7 | 90.6 | 42.9 | 65.2 | 14.2 | 13.7 | 3.0 | 4.8 |
| 7/6 –12 | 72.6 | 96.6 | 61.5 | 58.0 | 23.4 | 23.7 | 2.6 | 2.4 |
| 7/12–17 | 70.6 | 106.4 | 58.8 | 43.4 | 37.4 | 39.1 | 1.6 | 1.1 |
| 7/17–21 | — | 79.2 | — | 48.6 | — | 34.8 | — | 1.4 |
| 7/17–24 | 99.4 | — | 56.7 | — | 38.7 | — | 1.5 | — |
| 7/21–8/1 | — | 90.4 | — | — | — | — | — | — |
| 7/24–8/1 | 16.4 | — | — | — | — | — | — | — |

In the beginning the gas consisted chiefly of methane, the $CH_4/CO_2$ ratio being 8 or higher. However, from 6/22 onward, when the medium was not replaced any more, but a small volume of concentrated formaldehyde solution added, the percentage of $CO_2$ in the gas rose and the $CH_4/CO_2$ ratio decreased to about 1.5. This change is easily understood when it is borne in mind that $CO_2$ is much more soluble in water than $CH_4$ so that a certain amount of $CO_2$ remains in solution in the medium and is removed from the bottle at the replacements. When a medium with 1 % Ca-acetate is used this amount of $CO_2$ is less than half that which is produced by complete conversion of the substrate ¹), but when 0.1 % of

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¹) 250 ml of water of 30° C. will dissolve 166 ml of $CO_2$ at 1 atm., while 250 ml 1 % Ca-acetate, according to the equation:

$$Ca(OOCCH_3)_2 + H_2O = CaCO_3 + CO_2 + 2\,CH_4$$

will yield about 400 ml of $CO_2$ of 30° C. and 1 atm.

substrate is used the greater part of the $CO_2$ produced will remain in solution instead of escaping with the $CH_4$.

## § 4. Discussion.

The results mentioned in § 2 show that aldehyde-binding substances like hydroxylamine and phenylhydrazine indeed possess inhibitive power towards methane fermentation of acetate, similar to that of sulphite. However, dimedone, which is a quite specific reagent on aldehydes, is only slightly inhibitive. It is possible that the cells of the methane bacteria are less permeable to dimedone than to sulphite, hydroxylamine or phenylhydrazine, although the molecule of the latter is of about the same size as that of dimedone. Possibly the permeability is also influenced by the polarity of the molecules.

Hydrazines and hydroxylamines are known to influence biological systems in various ways. Warburg and coworkers (30) described reactions of phenylhydrazine and phenylhydroxylamine with the hemoglobin in erythrocytes. Lipmann and Tuttle (14) found that hydroxylamine reacts readily with the carboxyl group of acetylphosphate and employed this reaction for the demonstration of the production of acetylphosphate in animal tissues. According to Schales e.a. (21) proteolysis by pepsin, trypsin and papain is inhibited by small amounts of hydroxylamine, semicarbazide, hydrazines, bisulphite and dimedone, all typical carbonyl reagents. Wilson and coworkers (31, 32) investigated the action of hydroxylamine on the metabolism of *Azotobacter vinelandii*. Both nitrogen fixation and assimilation of ammonium nitrate appeared to be inhibited by very low concentrations (1—2 mg/l).

Therefore, although sulphite, hydrazine and hydroxylamine may act upon methane fermentation of acetate by combining with carbonyl groups of the enzyme system, other possibilities cannot be excluded.

Sodium azide is a rather powerful inhibitor of micro-organisms, about 0.01 % preventing the growth of a number of species of bacteria and fungi (cf. Lichstein and Soule (12) and Herrick and Kempf (5)). The former investigators report, moreover, that anaerobic bacteria like streptococci, clostridia and thermobacteria are distinctly less sensitive (not inhibited by 0.03 %). 0.01 % azide inhibited the catalase activity of washed bacteria. Keilin and Hartree (6) described a compound of catalase and azide, but state that the affinity of catalase for azide is much smaller than that for cyanide. STANNARD and HORECKER (26) obtained a similar compound of azide with cytochrome c and found an inhibition of cytochrome oxidase by azide. They state that the undissociated acid $HN_3$ forms a complex with the oxidase which is about as stable as the corresponding complex with HCN.

Besides for its action upon iron-porphyrin enzymes, azide is known for its inhibition of phosphorylation. BROCKMANN and STIER (1) suppressed the accumulation of energy-rich phosphate in alcoholic fermentation by the addition of $2.10^{-3}$ m azide and similar results were obtained by SPIEGELMAN, KAMEN and SUSSMAN (25). LOOMIS and LIPMANN (16) found the same inhibition in kidney homogenate. The inhibition of iron-porphyrin enzymes by cyanide is too well-known than that it needs to be discussed here. However, also inhibition of anaerobic metabolic processes by cyanide is mentioned in the literature. KEMPNER (7) gives the following figures for the inhibition of various processess in a Warburg apparatus:

| process | molarity of HCN | % inhibition |
|---|---|---|
| respiration of yeast | $10^{-4}$ | 40 |
| ,, ,, *E. coli* | $10^{-4}$ | 60 |
| ,, ,, *Vibrio Metschnikoff* | $10^{-3}$ | 80 |
| ,, ,, *Mycobacterium tuberculosis* | $10^{-3}$ | 50 |
| ,, ,, *Ps. aeruginosa* | $10^{-3}$ | 45 |
| alcoholic fermentation<br>lactic acid fermentation | $10^{-2}$ | 30—50 |
| butyric acid fermentation [1] | $10^{-4}$ | 23 |
| ,, ,, ,, | $10^{-3}$ | 66 |
| ,, ,, ,, | $10^{-2}$ | 100 |

KEMPNER also found an inhibition of butyric acid fermentation by carbon monoxide which, however, could not be reversed by illumination, unless very high intensities were employed (cf. (8)).

KUBOWITZ (10) discovered that carbon monoxide did not inhibit the conversion of sugar in this fermentation, but only the production of $CO_2$ and $H_2$, the sugar being converted to lactic acid. He further states that carbon monoxide has the same influence upon other anaerobic processes in which hydrogen is evolved, *viz.*, sugar fermentation and fission of formic acid by *E. coli* and fermentation of pyruvic acid by butyric acid bacteria. STEPHENSON and STICKLAND (27) mention 50 % inhibition by $10^{-5}$ m cyanide of the conversion of formic acid by *E. coli* and complete inhibition by an atmosphere of pure CO. KUBOWITZ found that $10^{-2}$ m cyanide also changed the butyric acid fermentation into a lactic acid fermentation. He further found that this cyanide concentration caused a 56 % inhibition of sugar utilization by *Clostridium butyricum*, the same holding for fermentation of lactic acid bacteria, tumors and yeast (compare KEMPNER's figures).

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[1] *Clostridium butyricum.*

A similar inhibition by CO was found by SCHLAYER (22) for the fermentation of tetanus and gas gangrene bacteria and by SIMON (24) for *Clostridium acetobutylicum*. Finally, PAPPENHEIMER and SHASKAN (20) could effect the same conversion of a butyric to a lactic acid fermentation in *Clostridium welchii* (*perfringens*) by growing the organism in a medium with a low iron concentration.

Thus, all the above-mentioned data clearly point towards the activity of an iron-porphyrin enzyme system in anaerobic processes in which hydrogen is evolved.

The great sensitivity of the methane fermentation of acetate to cyanide certainly points towards a rôle of a heavy metal catalyst in this process. However, it is by no means certain that an iron-porphyrin enzyme system is involved, as these (at least the respiratory ones) are about as sensitive to sulphide as to cyanide (cf. e.g. NEGELEIN (17), p. 206), whereas methane fermentation of acetate is only slightly sensitive towards this substance. Unfortunately, the above-mentioned investigators did not determine the sensitivity of their hydrogen-evolving enzyme systems towards sulphide. Carbon monoxide could not be included in the present investigation, but it seems doubtful whether this inhibitor will have any action upon methane fermentation of acetate: the work of LIESKE (13) and of FISCHER and coworkers (4) and of KLUYVER and SCHNELLEN (9) clearly shows that methane-producing bacteria exist which can convert CO (both in the presence and in the absence of $H_2$) to methane.

The inhibition of methane fermentation of acetate by azide might be due to action upon a heavy metal catalyst, but also to interference with a phosphorylation process; the same might hold for the action of hydroxylamine. Nothing definite is known yet of a rôle of phosphate in methane fermentation, it is true, but there are good reasons to expect it on general biochemical grounds.

The results obtained with formaldehyde leave no doubt that the bacterial population in the acetate cultures employed contained organisms which were able to convert this compound to methane and carbon dioxide, presumably according to the equation:

$$2\ CH_2O = CH_4 + CO_2 \ ^{1)}$$

The total amount of formaldehyde added to each culture on the dates 3/29—7/13 '50 was about 1500 mg, i.e. 50 mmols. The volumes of methane produced from this amount were approximately 2):

$$\left.\begin{array}{lll}\text{Culture} & \text{I} & 290\ \text{ml}\\ ,, & \text{II} & 360\ ,,\end{array}\right\}\ \text{of } 30^\circ\ \text{C.}$$

According to the equation mentioned above these amounts are equivalent to

| | | | | | |
|---|---|---|---|---|---|
| Culture | I | 23 | mmols | of | $CH_2O$ |
| ,, | II | 29 | ,, | ,, | ,, |

Therefore, at least a large part of the formaldehyde added can be accounted for in this way.

Formaldehyde is one of the strongest bactericides known, 0.0001 % killing many species of bacteria (cf. BUCHANAN and FULMER (2), p. 433). However, there are a couple of references to a higher resistance: LAMPE and ROEHRICH (11) found yeast growing on potato mash resistant to 0.02 % and THOMPSON and DODD (29) succeeded in accustoming *Corynebacterium diphtheriae* to develop in the presence of even 0.2 %, whereas the unadapted organism did not grow with 0.006 %.

Utilization of formaldehyde or derivatives is mentioned in three instances. LOEW (15) isolated an asporogenous aerobic rod from a mineral medium containing 0.5 % Na-formaldehyde-bisulphite and NEUBERG and WELDE (18) observed a reduction of formaldehyde to methanol by a yeast suspension in vigorous alcoholic fermentation. STEPHENSON and STICKLAND (28) obtained a culture of methane bacteria which fermented formate to $CH_4$ and $CO_2$ (but no compounds with more than one C atom) and which could reduce

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1) The theoretical $CH_4/CO_2$ ratio of 1 is approached in the last 4 analyses mentioned in table III.

2) Calculated from analyses of 4/14—7/24.

$CO_2$, formic acid, CO, formaldehyde (in the form of hexamethylenetetramine) and methanol with gaseous hydrogen to methane. However, SCHNELLEN (23) could not detect any conversion of hexamethylenetetramine (or methylal, $CH_2(OCH_3)_2$) by *Methanobacterium formicicum* and *Methanobacterium Omelianskii* or *Methanosarcina Barkeri*, all species which are active in the hydrogenation of $CO_2$ to $CH_4$ with $H_2$.

That certain methane bacteria are able to ferment the generally very toxic free formaldehyde in concentrations up to 0.15 % can be explained by assuming that the formaldehyde, when penetrating into the cell, is converted immediately to methane and carbon dioxide. An interesting parallel to this behaviour among methane bacteria is the ability of sulphate-reducing bacteria to thrive in media containing up to 5 % of $Na_2SO_3 . 7aq$, although 0.1 % sulphite inhibits the growth of most micro-organisms (cf. BUTLIN e.a. (3)). These bacteria possess a very efficient "detoxicating" mechanism in their enzyme system for the reduction of sulphur compounds to $H_2S$.

It is evident that more questions are posed than answered by the present investigation. It is the authors' hope that somebody else will be able to pursue the investigations upon inhibition of methane fermentation in a more elaborate way, if possible with pure cultures.

## Summary.

1. Methane fermentation of Ca-acetate is about as sensitive to phenylhydrazine, hydroxylamine and azide as it is to sulphite (inhibition by $10^{-2}$ m 70, 83, 94 and 95 % resp.; by $10^{-3}$ m 0, 39, 49 and 31 % resp.).
2. The sensitivity to dimedone is much less, $10^{-2}$ m inhibiting 17 % only.
3. The process is much more sensitive to cyanide, $5.10^{-4}$ m giving already ± 90 % inhibition.
4. The sensitivity to sulphide is much less, as $5.10^{-2}$ m is necessary for complete inhibition, whereas $10^{-2}$ m does not inhibit at all.
5. Cultures of methane bacteria on Ca-acetate can be accustomed to 0.15 % formaldehyde which is fermented to $CH_4$ and $CO_2$ in presumably equimolecular proportions.
6. The results are discussed in the light of data on the action of

these toxic substances in biological systems in general, with special reference to anaerobic bacteria.

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# STUDIES ON HAEMAGGLUTINATION BY THE EMC-MM-COLUMBIA SK-GROUP OF VIRUSES

## IV. EXPERIMENTS WITH EGG-ADAPTED VIRUS ¹)

by

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MM virus could be maintained in the chick embryo during 6 and 10 passages by BRUTSAERT, JUNGEBLUT and KNOX (4) and by ENRIGHT and SCHULTZ (5) respectively, whereas Columbia SK virus was maintained in 30 egg passages by ENRIGHT and SCHULTZ (5). Since these investigators obtained mouse infectivity titres of $10^{-6}$ and $10^{-7}$ per ml, and HALLAUER (7) observed haemagglutination of sheep red cells with egg-adapted virus if the mouse infectivity titre was at least $10^{-4}$ to $10^{-5}$, the use of egg material might be more advantageous than infected mouse brain suspensions. Moreover, a haemagglutination inhibitor, which has been shown in mouse brain by DE BAAN, VERLINDE and WALLER-FETTER (2) seems to be absent in extra-embryonic fluids of the chick embryo.

The present paper deals with the results of mouse infectivity titrations and haemagglutination reactions carried out with egg-adapted MM and Columbia SK virus.

It appeared from preliminary experiments, that 8 to 10 day old embryonated eggs were most suitable. Then, two series of egg passages were made. In one series, the virus (1 per cent centrifuged mouse brain suspension) was passed by inoculation on the chorio-allantoic membrane, in the second the passages were made by amniotic inoculation. The eggs were incubated for 4 days at 35° C. following inoculation. Embryos that died within 4 days were discar-

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¹) Third publication: Antonie van Leeuwenhoek **17**, 137, 1951.

ded, since the virus concentration in these materials appeared to be less than in living embryos of the same age. A longer period of incubation did not result in a higher concentration of virus.

The haemagglutination reactions were carried out by the method described earlier (1, 2), and using Krebs solution as a diluent.

Chorioallantoic inoculation.

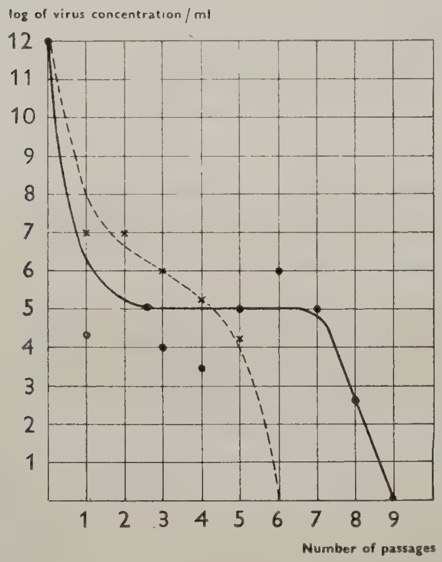

Fig. 1. Mouse infectivity titrations of allantoic fluid and chick embryo brain after chorioallantoic passages of MM virus.

———— allantoic fluid.

– – – – – chick embryo brain.

As can be seen from figure 1, the MM virus maintained itself in the allantoic fluid during 7 chorioallantoic passages at approximately the same level, which was far less than the ID50 titre of the original mouse brain suspension. After the 7th passage, however, the ID50 titre decreased rapidly, and in the 9th passage no virus could be demonstrated in the allantoic fluid by mouse inoculation. The infectivity titre of the embryonic brain tissue showed a more rapid fall, and the virus had disappeared in the 6th passage already.

Columbia SK virus disappeared from the allantoic fluid in the 6th passage, when transmitted from egg-to-egg by chorioallantoic inoculation.

No haemagglutination was observed when mixing twofold dilutions of allantoic fluid with washed sheep erythrocytes, which proved agglutinable by virulent mouse brain suspension. Suspensions of embryonic brain, however, produced a weakly positive haemagglutination to a maximum titre of 1/48, whereas brain suspensions of normal chick embryos were constantly negative.

Amniotic inoculation.

Fig. 2 shows, that the mouse infectivity titre of amniotic fluid infected with MM virus, and with Columbia SK virus as well, may gradually increase during 21 amnion passages to a ID50 titre of $10^{-9}$ per ml, but that the titre of the original mouse brain has not been reached. The virus also proved to be present in the allantoic fluid, but approximately to the same titre or one log higher as that obtained following chorioallantoic inoculation. When the amniotic fluid was passed by inoculation into the allantoic sac, the first passage resulted in a higher titre of the allantoic fluid ($10^{-8}$ to $10^{-10}$) than could be obtained by passing mouse brain suspension by chorioallantoic inoculation. Subsequent allantoic passages, however, resulted in a rapid decrease of the mouse infectivity titre, and the virus was completely lost after a few passages.

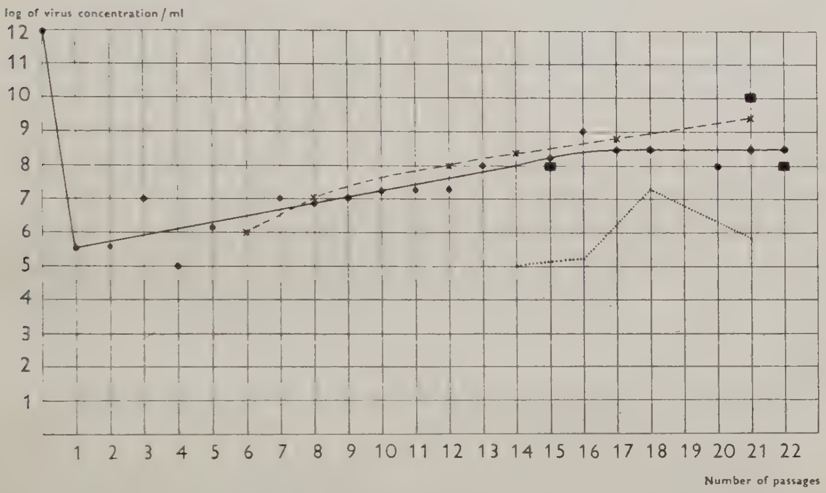

Fig. 2. Mouse infectivity titrations of amniotic fluid, allantoic fluid, and edema fluid after amniotic inoculation of MM virus.

——— amniotic fluid.

– – – – – edema fluid.

……………… allantoic fluid after amniotic inoculation.

■ allantoic fluid after inoculation of amniotic fluid into the allantoic sac.

Hemorrhages and a marked subcutaneous edema of the chick embryos were regularly observed following amniotic inoculation. The edema fluid appeared to have approximately the same ID50 titre as the amniotic fluid.

In order to examine the distribution of the MM virus in the chick embryo, the fluids and embryonic tissues of the 21st amnion passage were tested for the presence of virus and for its mouse infectivity. As can be seen from table 1, the virus is present throughout the embryo.

TABLE 1.
Distribution of MM virus in embryos of the 21st amnion passage.

| Material tested | ID50 per ml |
|---|---|
| allantoic fluid | $10^{-6}$ |
| amniotic fluid | $10^{-8}$ |
| edema fluid | $10^{-9}$ |
| brain | $10^{-6}$ |
| heart | $10^{-5}$ |
| liver | $10^{-5}$ |
| spleen | $10^{-7}$ |

In spite of the high mouse infectivity titres of the fluids, no haemagglutination of sheep red cells could be produced. The maximum haemagglutination titre of embryonic brain tissue was 1/48, and that of whole edematous embryos was 1/32. The haemagglutination produced by the whole embryo appeared to be non-specific, since the same titres were obtained with suspensions of normal chick embryos of the same age.

Haemagglutination reactions with chick red cells and human O erythrocytes were negative.

Attempts have been made to explain the inability of the chick embryo materials to produce haemagglutination. Since we have shown in previous experiments (2) that in mouse brain a substance inhibiting haemagglutination of sheep erythrocytes is present, whereas allantoic fluid may stimulate rather than inhibit the haemagglutination, the assumption of the presence of a factor inhibiting the haemagglutination of sheep red cells, comparable to the non-specific inhibitor of influenza haemagglutinin, in extra-embryonic fluids does not seem to be justified.

Nevertheless, we have tried to neutralize a possible inhibitor with a filtrate of a *Vibrio cholerae* culture (VAN DER VEEN and MULDER (9)) according to the method employed by SCHLESINGER (8), who succeeded in unmasking influenza haemagglutinin in brain suspension of mice inoculated intracerebrally with influenza virus by digestion with cholera enzyme (RDE). Therefore, infectious amniotic and allantoic fluids were digested with cholera filtrate containing potent RDE, for 12 hours at 37° C., and for 12 hours at 37° C. followed by destruction of the enzymatic activity by heating for 1 hour at 56°C. In spite of these treatments, Columbia SK virus-infected extra-embryonic fluids failed to produce haemagglutination of sheep red cells, and with infected mouse brain suspensions similarly treated, no increase in haemagglutination titre was observed when compared with control mouse brain suspensions digested without cholera filtrate.

It has been shown previously (10), that adsorption of the virus to sheep red cells, and the presence of certain electrolytes (GARD and HELLER (6), BREMER (3)) are indispensable for the haemagglutination. As to the latter, Krebs solution containing several electrolytes (sodium chloride, potassium chloride, calcium chloride, potassium biphosphate, magnesium sulphate, and sodium bicarbonate), as well as veronal saline have been used as diluents.

The adsorption of the virus present in extra-embryonic fluids to sheep red cells was examined by mixing 3 ml of infective amniotic fluid and 3 ml of a 50 per cent sheep red cell suspension. The mixture was placed at 4° C. for 3 hours, and then centrifuged at 3000 r.p.m. for 30 minutes. Infected amniotic fluid of the same batch was placed at 4° C. for 3 hours and centrifuged, without adding red cells. The supernatant fluid from both tubes was tested for infectivity by intracerebral inoculation of mice. The ID50 of the control fluid was $10^{-6}$, that of the virus-red cell mixture was $10^{-3}$, which indicates, that adsorption of the virus to the red cells has occurred, but to a degree far less than the virus from mouse brain is adsorbed.

This result suggests, that the concentration of virus adsorbed to the red cells, is important for the initiation of the haemagglutination. If only a certain percentage of the virus is adsorbed, it is obvious, that haemagglutination occurs when the concentration of virus in the material is extremely high, and this is, as a rule, not the case when infected egg fluids are used. Besides, another factor which is likely to be present in mouse brain and absent in egg fluids, seems to play a rôle, since the virus in mouse brain is always nearly completely adsorbed. Haemagglutination seems to be effected by a number of coinciding factors. It may be assumed, that the inflammatory processes in the central nervous system, which are accompanied by biochemical alterations, have something to do with the haemagglutinating ability, but BREMER's (3) suggestion, that the haemagglutinating principle itself would be present in the central nervous system lesions only, does not seem most likely. We are inclined to consider the central nervous system lesion merely as one of the coinciding factors, which are necessary for the initiation of the haemagglutination.

## Summary.

When MM and Columbia SK virus were propagated in the chick embryo, the virus disappeared from the allantoic fluid and from the embryonic brain tissue after 6 to 9 chorioallantoic passages. Amniotic passages, however, resulted in a gradual increase of the mouse infectivity titre of the amniotic fluid and of the fluid of the regularly observed subcutaneous edema of the embryos, though after 22 passages the ID50 titre was still 3 logs lower than that of the original mouse brain suspension. The virus was present not only in amniotic fluid, but also in allantoic fluid and throughout the whole embryo. In spite of the pretty high mouse infectivity titres, the extra-embryonic fluids failed to produce haemagglutination of sheep red cells, whereas low haemagglutination titres could be obtained with suspensions of embryonic brain tissue. No evidence was obtained, that a non-specific inhibitor was responsible for the inability to produce haemagglutination. The virus present in egg fluids is only partially adsorbed, which is in contrast with the almost complete adsorption of virus in mouse brain suspension. The adsorption of a relatively small amount of virus might be one of the factors responsible for lack of haemagglutinating ability. It is suggested, that another coinciding factor, which is necessary for the initiation of the haemagglutination might be present in the central nervous system lesion.

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(Laboratory for Flowerbulb-research at Lisse).

# DIAGNOSIS OF PLANT DISEASES BY ELECTRON-MICROSCOPY

by

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(Received February 22, 1951).

The electron microscope can be a great help for the diagnosis of plant diseases, for which purpose our Institute has been supplied with this instrument. In many cases it has made possible the identification of viruses present in suspected plants.

Regarding KOCH's postulates, however, the electron microscope may enable us to cope with the necessity to show heretofore unvisible parasitical agents in the host; still more important is the application of all of KOCH's postulates to the object that is seen under the electron microscope and is supposed to be the virus.

The new Philips electron microscope has proved to be reliable enough for us to master most of the difficulties of the technique of electron microscopy as far as the instrument itself is concerned ¹). The main difficulties have arisen with the making of the preparates and those depend on the nature of the object to be investigated.

The electron microscope may open a new field of research, but once again it must be evident that no instrument, however perfect and valuable it may be, will alone as a "deus ex machina" solve the problems. It certainly will serve its purpose best, if it can supply the missing link in the chain of many more facts needed for the

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¹) We have to acknowledge that for the high quality of the photographs in this article we are very much indebted to our Mr. J. D. BAKKER. Only by his ability and devotion have we been able to overcome many difficulties. All other members of our staff have also given their full cooperation for the handling of the material for the preparates.

solution of the problems. How much of the picture seen on the screen of the electron microscope or in the photographs may be an artefact? How are the size and the shape of the particles modified by the different treatments of the material?

Especially in this first stage of the use of the electron microscope it seems important to us that the experiences of the different laboratories be exchanged. It also seems necessary that, together with the photographs, there should be some indication as how the material has been treated.

Up to now we have investigated some bacteria that cause diseases of flowerbulbs and a number of viruses that play havoc with several horticultural and agricultural crops. Bacteria are not too difficult to recognize in a picture and to identify, if it is known from research with the ordinary microscope, what may be expected. The only difficulty may be to get them in full dress in the picture, with the cilia to make identification possible. The electron microscope may even save time for identification if the necessity to make pure cultures by isolation can be avoided.

The figures 3 and 4 show *Xanthomonas hyacinthi* (Wakker) Dowson, the cause of the Yellow disease of hyacinths, and fig. 5 shows *Bacterium carotovorum*, a wound parasite of hyacinths.

More difficult is the identification of viruses. Often there may be found in the juice of a diseased plant some particles that might be a virus, but too often the same particles will be found in the juice of other plants, that have been considered as perfectly healthy. Even then it may be some other virus that is carried by both types of plants without causing any symptoms of a disease, and not the virus under investigation.

In other cases it has not been possible to find anything with even the suggestion of a virus particle in the preparates made with the juice of severely diseased plants. This may be due to the fact that the concentration of the virus is very low, or rather that by the different treatments applied to the plant juice for the purification and concentration of the virus, it has been lost. Sometimes the juice has given a positive serological reaction with the specific antiserum of the virus, and even then it was not possible to discover any virus particle in the photograph. Sometimes it has been very difficult to separate the virus from the normal protoplasm of the host and this coincides with our experience from serological research in cases where it was very difficult to separate the virus from the plastids, either by ultracentrifugation or by precipitation with different chemicals.

In some cases (figs. 14 and 16) it has been possible to show the agglutination of the virus particles after adding the specific antiserum made against the virus under investigation. With addition of normal-serum no agglutination of the particles could be seen.

In other cases it could be proved that a suspected contamination with another virus was indeed present. E.g. some tobacco plants gave a positive serological reaction with the antiserum against tobacco necrosis, and not with the specific tobacco mosaic antiserum. Other plants reacted positively with tobacco mosaic antiserum and not with tobacco necrosis antiserum.

The first plants showed in their juice the virus particles of tobacco necrosis (round) and the others the particles of tobacco mosaic virus (rods).

Two plants that did not show symptoms of the tobacco mosaic disease, but which reacted positively with both antisera, showed in their juice both the round and the rod-shaped virus particles.

Dr F. C. BAWDEN mentions in the third edition of his book on Plant Viruses that the virus particles of cucumber mosaic have the shape of a rod. We obtained from Mr. NOORDAM (Aalsmeer) a virus strain from Chrysanthemum, transmitted to tobacco and thought possibly to be a cucumber virus. In the juice we found only round-virus particles. Later we received a strain of cucumber virus I from Dr TJALLINGII (Venlo) and after transmission to tobacco we again could find only round-particles that might be a virus. No rod-shaped particles could be seen. For the present we accept the round-particles to be those of cucumber virus I, that causes the virus disease of cucumber and other plants in our country.

More exchange of experiences may solve this controversy, if it exists. If both viruses are strains of a cucumber virus, then it would be shown that different strains of a virus could differ widely in form. The difference seems too great, however, to accept this fact without a thorough investigation.

## METHOD.

Concerning the technique of our making the preparates: The objectholder of our instrument is a small silver plate with a slit 1000$\mu$ long and 150$\mu$ wide. This slit is covered with a thin film of "formvar" [polyvinyl formal dissolved in $^1/_4\%$ solution of dioxane (ethylene-dichloride)]. Later we were better satisfied by dissolving the formvar in $^1/_4\%$ solution of pure chloroform. Mr. BAKKER designed a method of correctly covering the objectholders with the formvar-film by placing them on a piece of monel-gauze, that had been thoroughly cleaned in ether to remove all grease. The holders are brought under the film, which is spread on the water in a culture dish, and by raising them slowly out of the water all are covered with a thin film closing the slit.

The plant juice is put on this film. Shadow casting mostly has been done with Palladium and sometimes with Gold-manganine, (50% — 50%).

Gevaert Duplo-ortho film gave good results, but sometimes was clouded by electric discharges. Therefore Gevaert made for our institute a special film: Scientia Nr. 39B50, 35 mm wide, without perforations. It is exposed for 5—10 seconds, depending on the voltage used and the object. The films have been developed in Nogranol fine grain developer. From the negative an enlarged (about 3 ×) positive is made on Gevaert Superchrom; in many cases Gevaert Dipos rapid film has given still better results. Prints and enlargements have been made on Gevaerto or on orthobrom-paper.

The most essential details about the handling of the material used, are mentioned with each print of the electron-microscopical photograph. The distance of the white tabs on each photograph is one $\mu$.

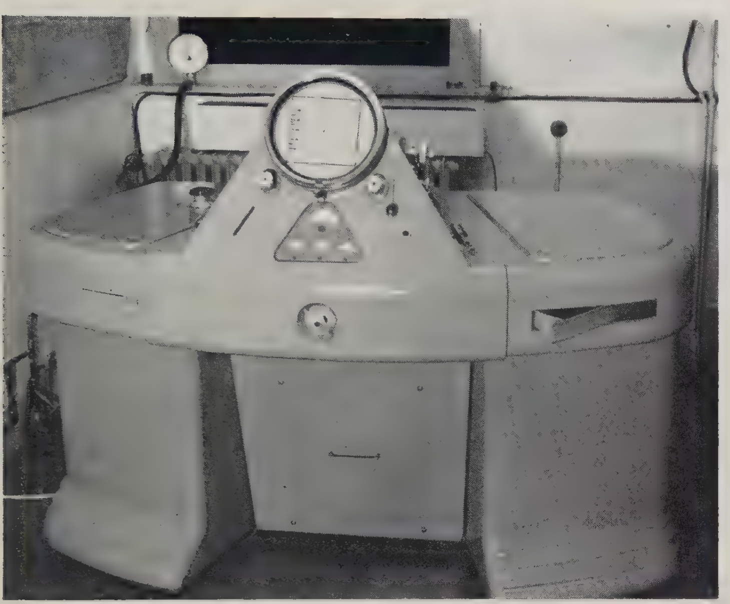

Fig. 1.
Philips Electron microscope model: Metalix no. 11980.

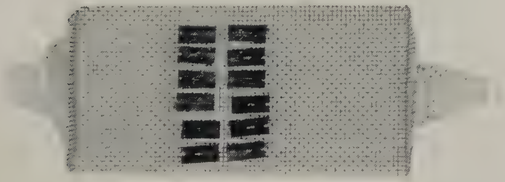

Fig. 2.
Monelgauze with objectholders.

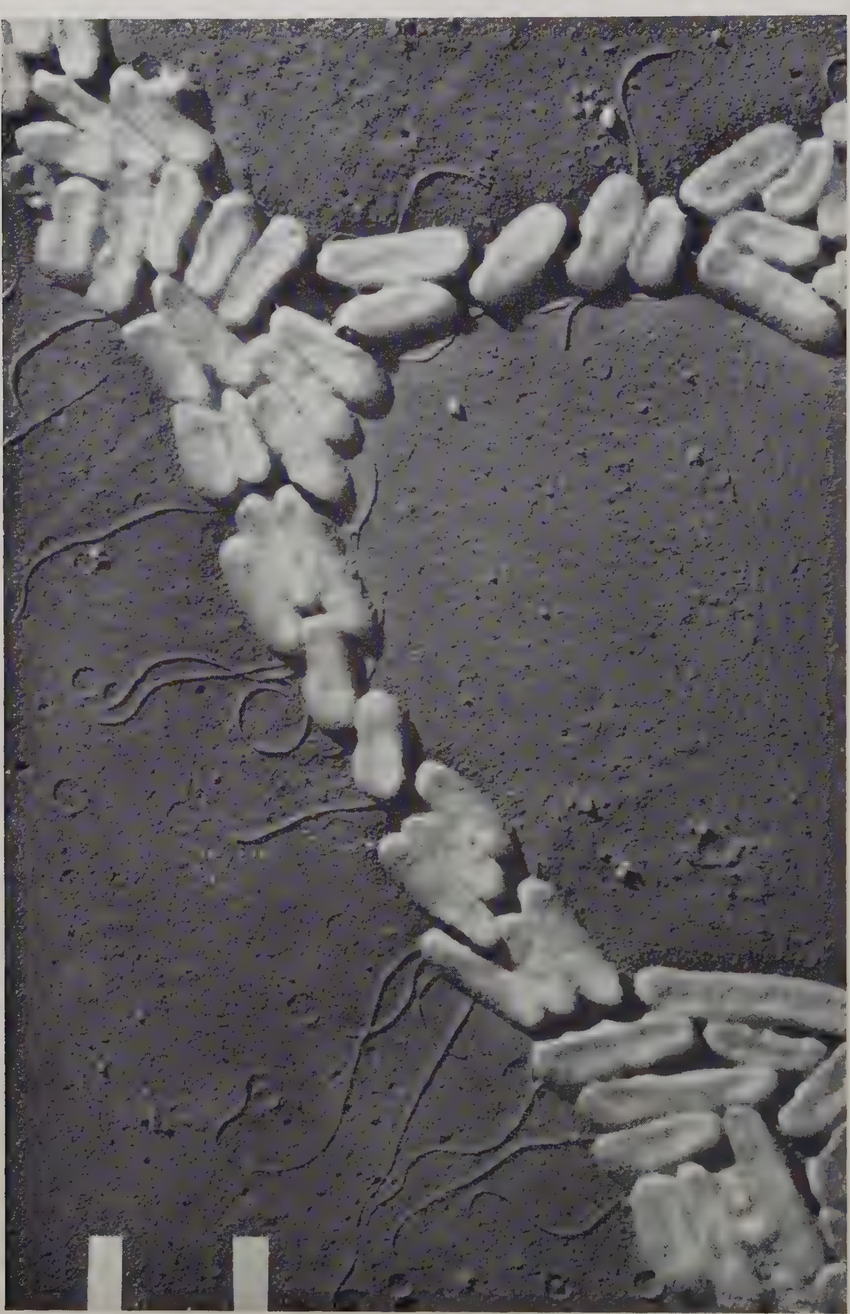

Fig. 3.
*Xanthomonas hyacinthi* (Wakker) Dowson.

Plant-tissue with bacteria in destilled water.
Shadowcast Palladium.
Enlargement 15000 × . Photograph 5.6.'50 (E.f.19).

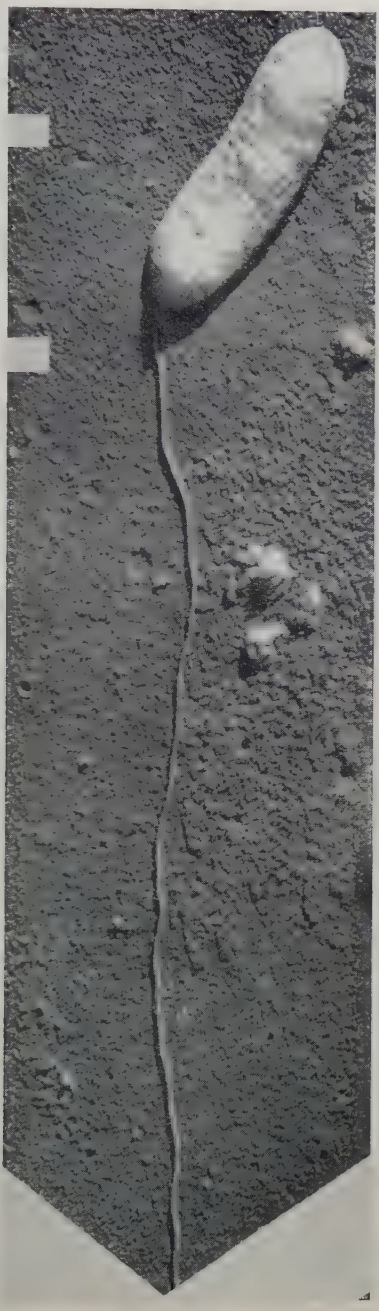

Fig. 4.
*Xanthomonas hyacinthi* (Wakker) Dowson.

Material the same as in fig. 3.
Shadowcast Palladium.
Enlargement 25000 ×. Photograph 5.6.'50 (E.f.21).

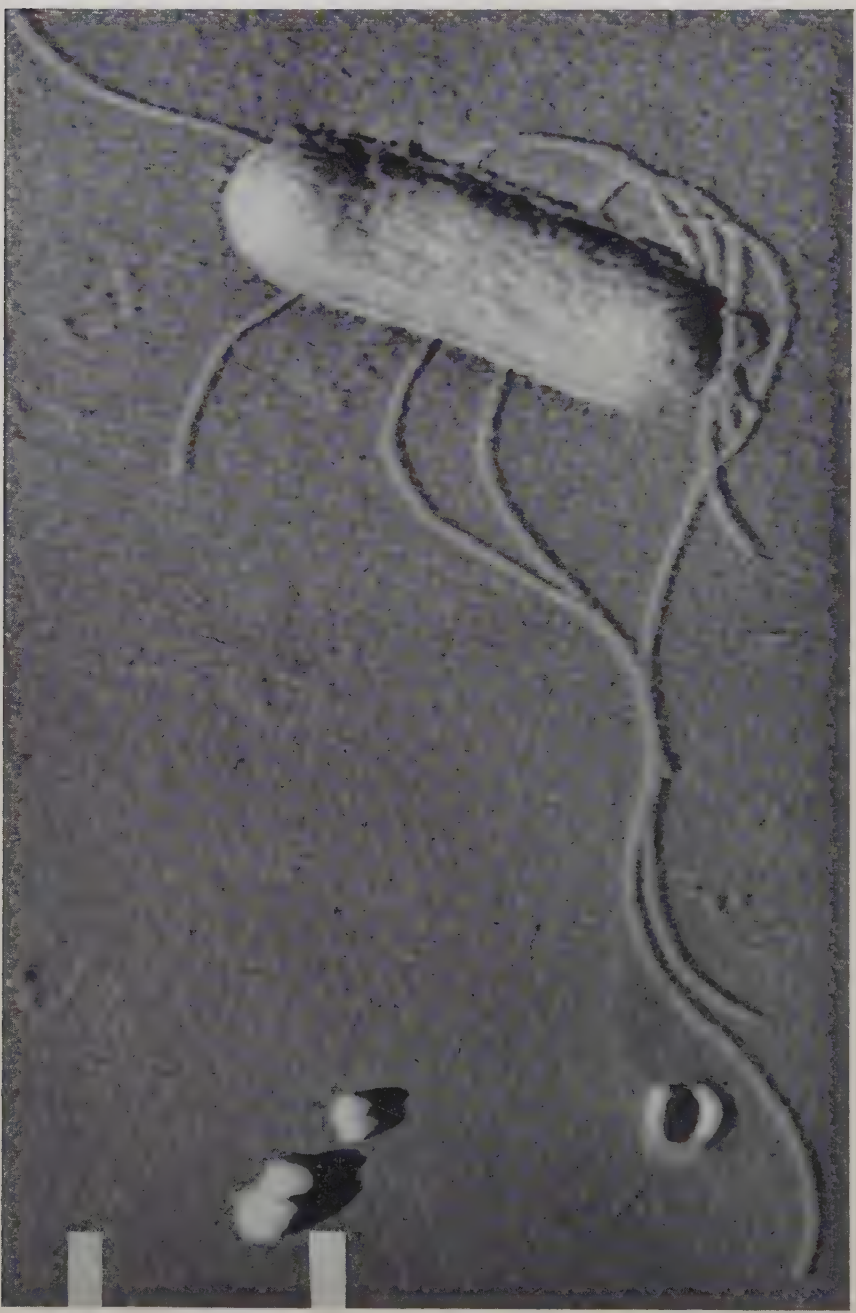

Fig. 5.

*Bacterium carotovorum* (Jones) Lehmann et Neumann.

Material from a leaf of Hyac. Arentine Arendsen, mixed in water.
Shadowcast Gold-manganine.
Enlargement 27000 ×. Photograph 29.12.'50 (E.f.142).

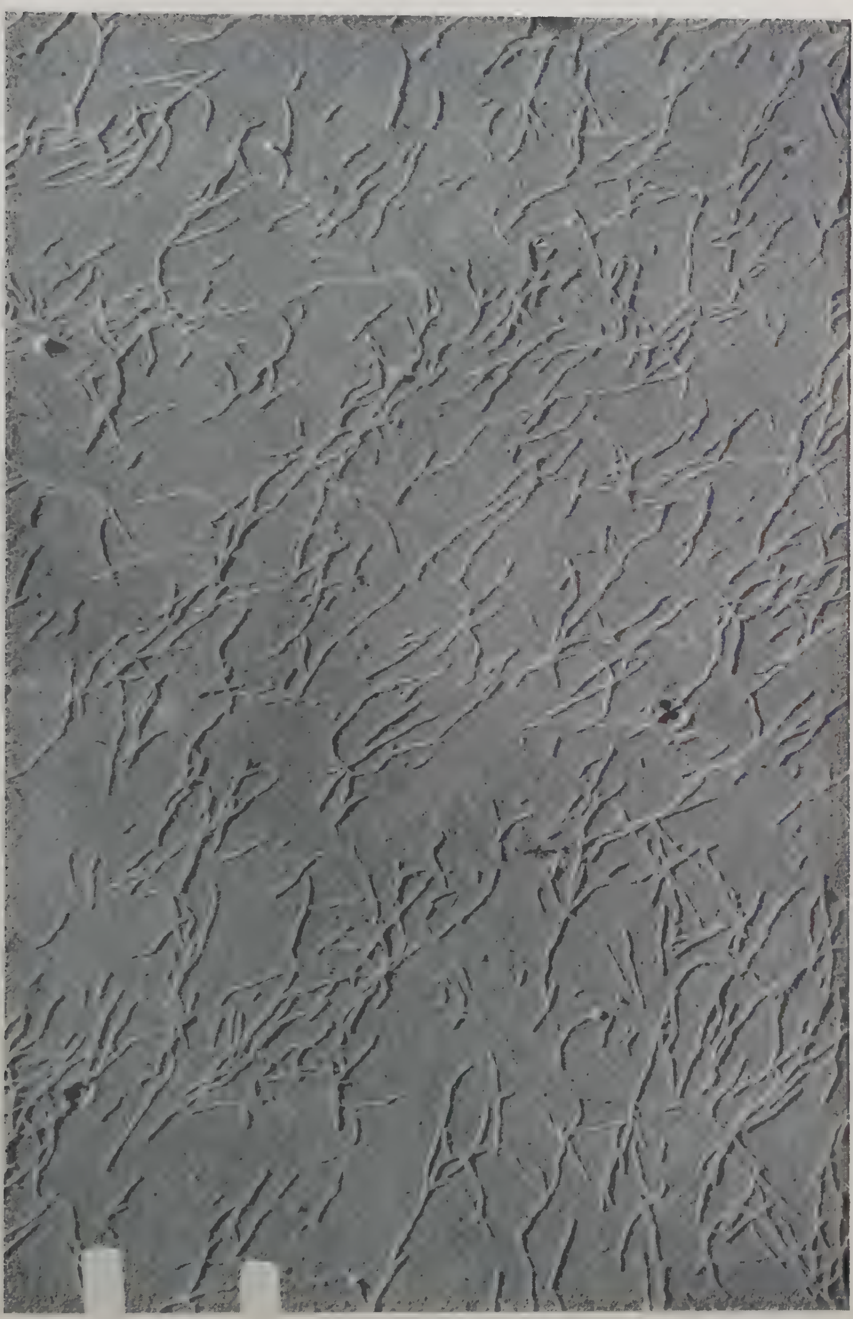

Fig. 6.
Potato X-virus.

Shadowcast Palladium.
Enlargement 15000 ×. Photograph 18.9.'50 (E.f.66).

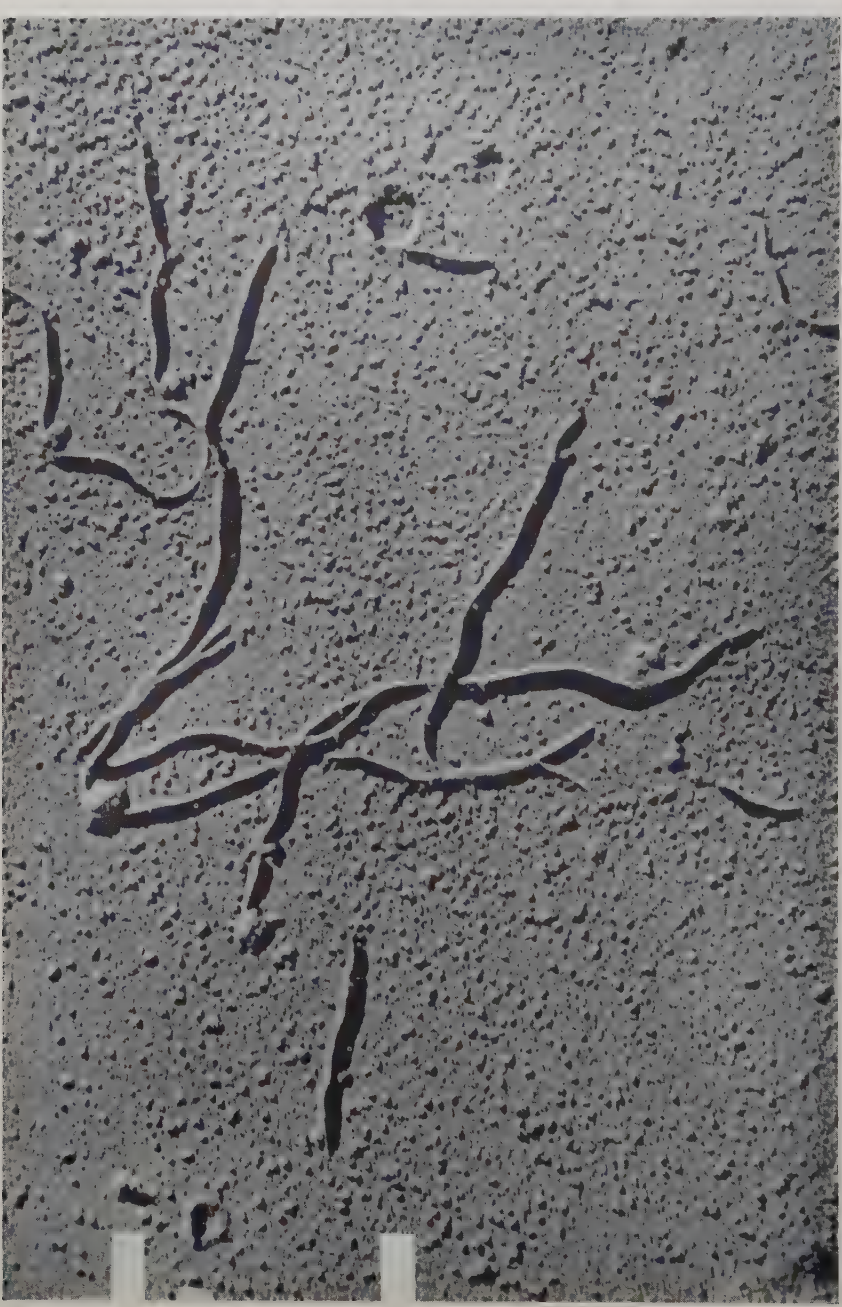

Fig. 7.
Potato Y-virus from tobacco.

Plants ground in meat-grinding machine — hydraulic press — ammonium sulfate — centrif. 90 min. 45.000 rot. — in shaker for 30 min. — $Na_2HPO_4$ — 30 % ammonium sulfate pH 7 — in shaker for 15 min. — ± 14% ammonium sulfate twice concentrated — dialyse. Shadowcast Palladium.
Enlargement 31000 ×. Photograph 16.2.'51 (E.f.170).

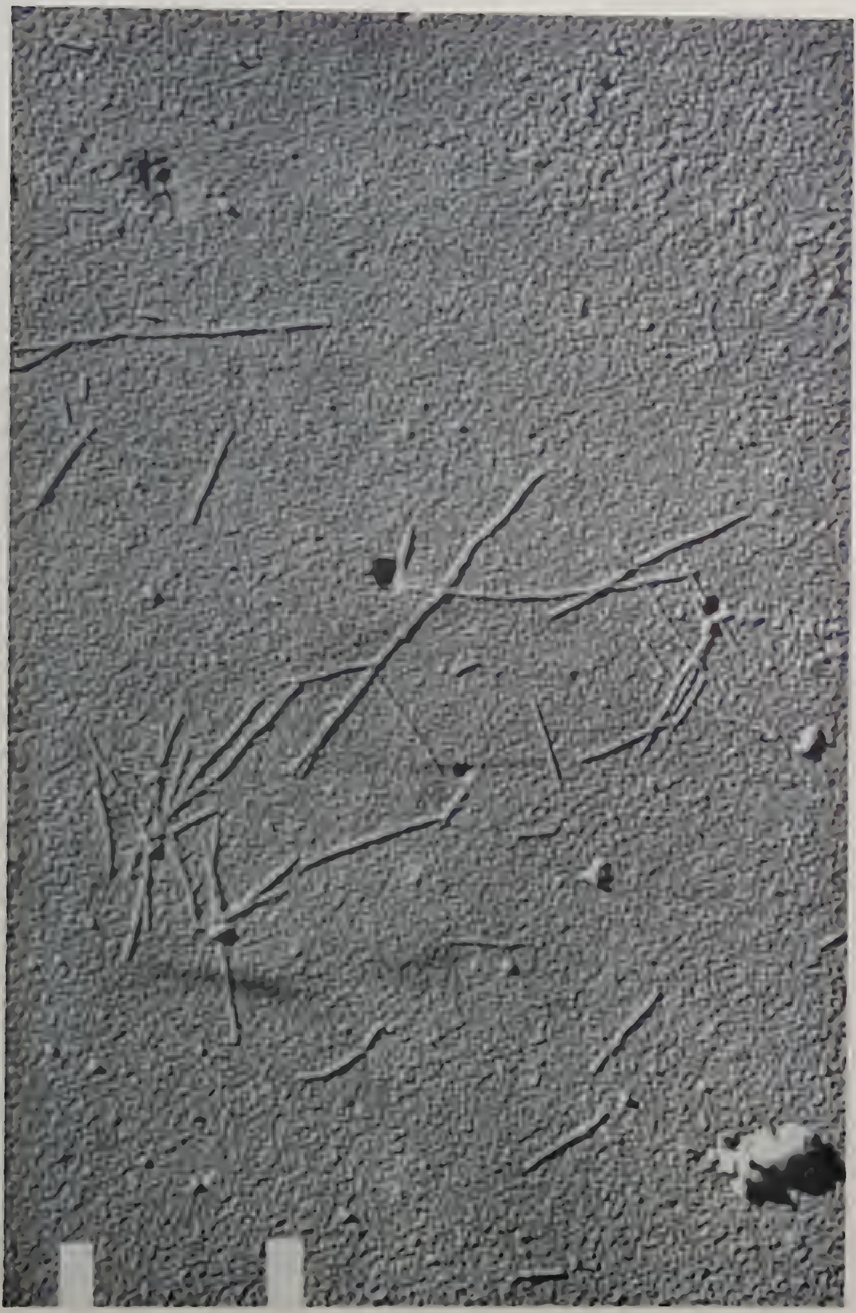

Fig. 8.

Tulip virus I from current season symptoms from petals of Darwin tulips Princess Elisabeth.

Squeezed in triple mill — purified with ammonium sulfate — centrif. 45 min. at 40.000 rot. — dialysed one night.
Shadowcast Palladium.
Enlargement 22000 ×. Photograph 2.8.'50 (E.f.44).

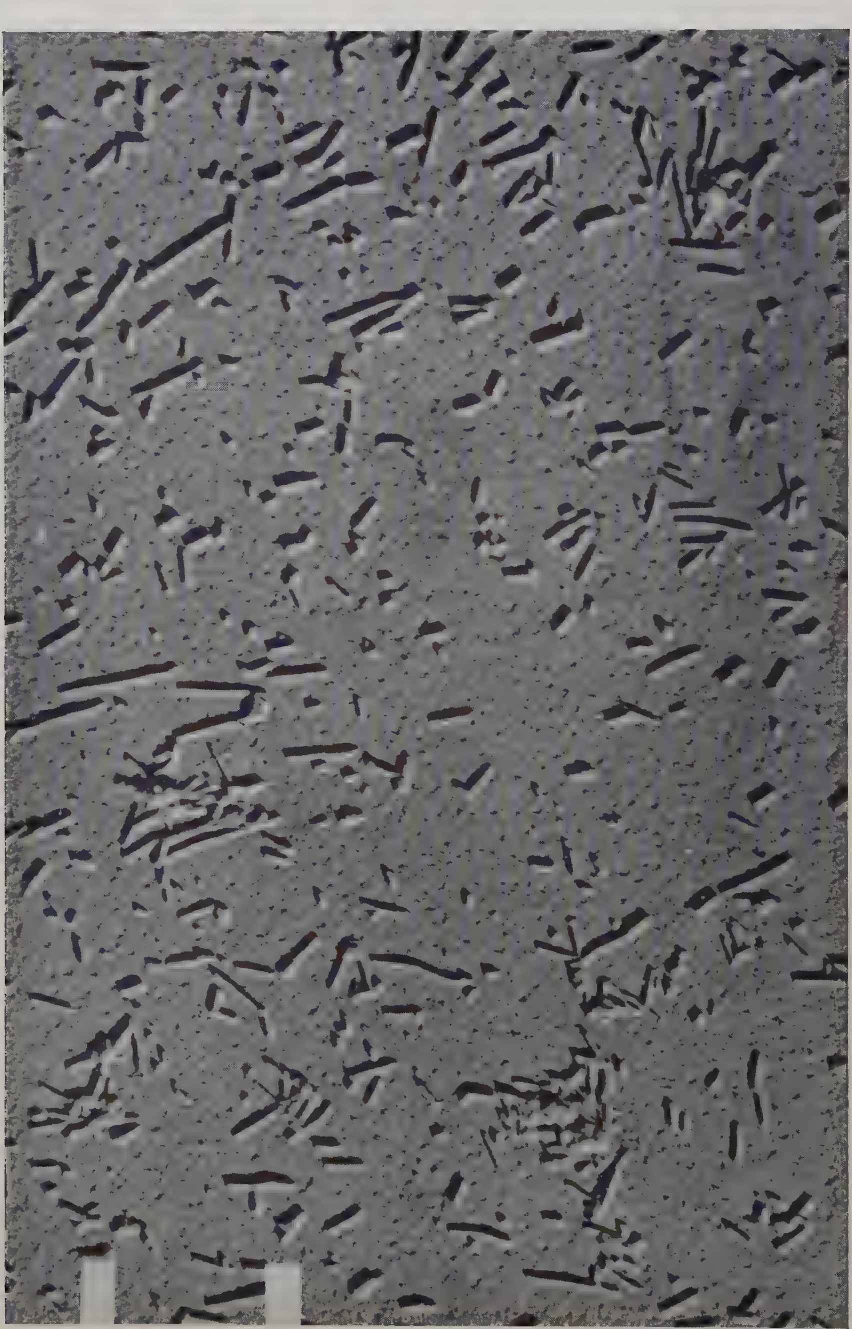

Fig. 9
Unknown virus from tulips that show whitish spots. Transmitted to tobacco. This may be the ratel virus of tobacco.

Triple mill — purified with ammonium sulfate — centrif. 60 min. at 40.000 rot. — dialysed one night.
Shadowcast Palladium.
Enlargement 20.000 ×. Photograph 8.11.'50 (E.f.89).

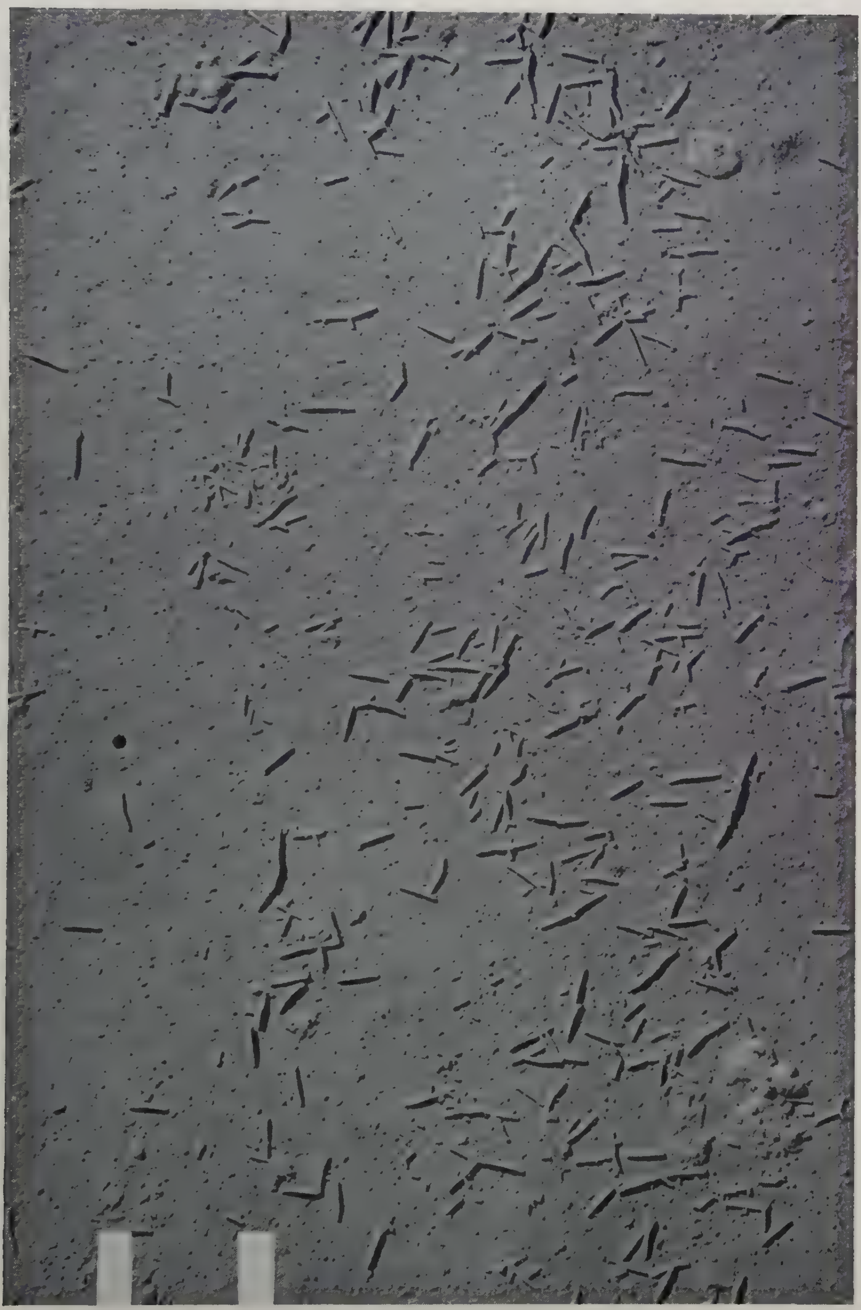

Fig. 10.
Unknown virus from Daffodils, transmitted to tobacco.

Triple mill — hydraulic press — centrif. 2 × 15 min. at. 8000 rot. and 60 min. at 40.000 rot. — dialysed for 16 hours.
Shadowcast Palladium.
Enlargement 14000 ×. Photograph 20.11.'50 (E.f.99).

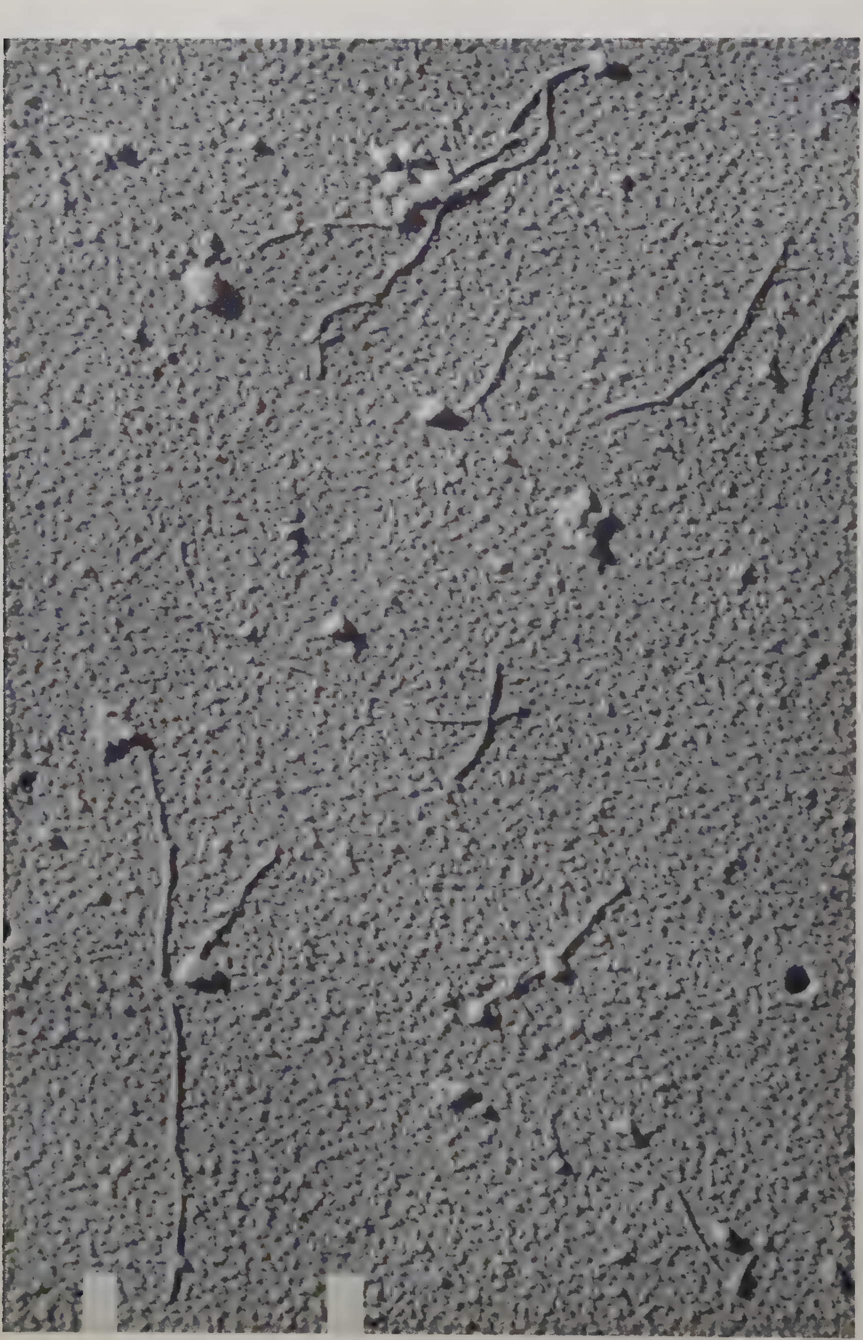

Fig. 11.
Phaseolus virus II.

Hydraulic press — ammonium sulfate — Sharpless centrif. 120 min. at 45.000 rot. — dialysed 10 hours.
Shadowcast Palladium.
Enlargement 27500 ×. Photograph 9.9.'50 (E.f.58).

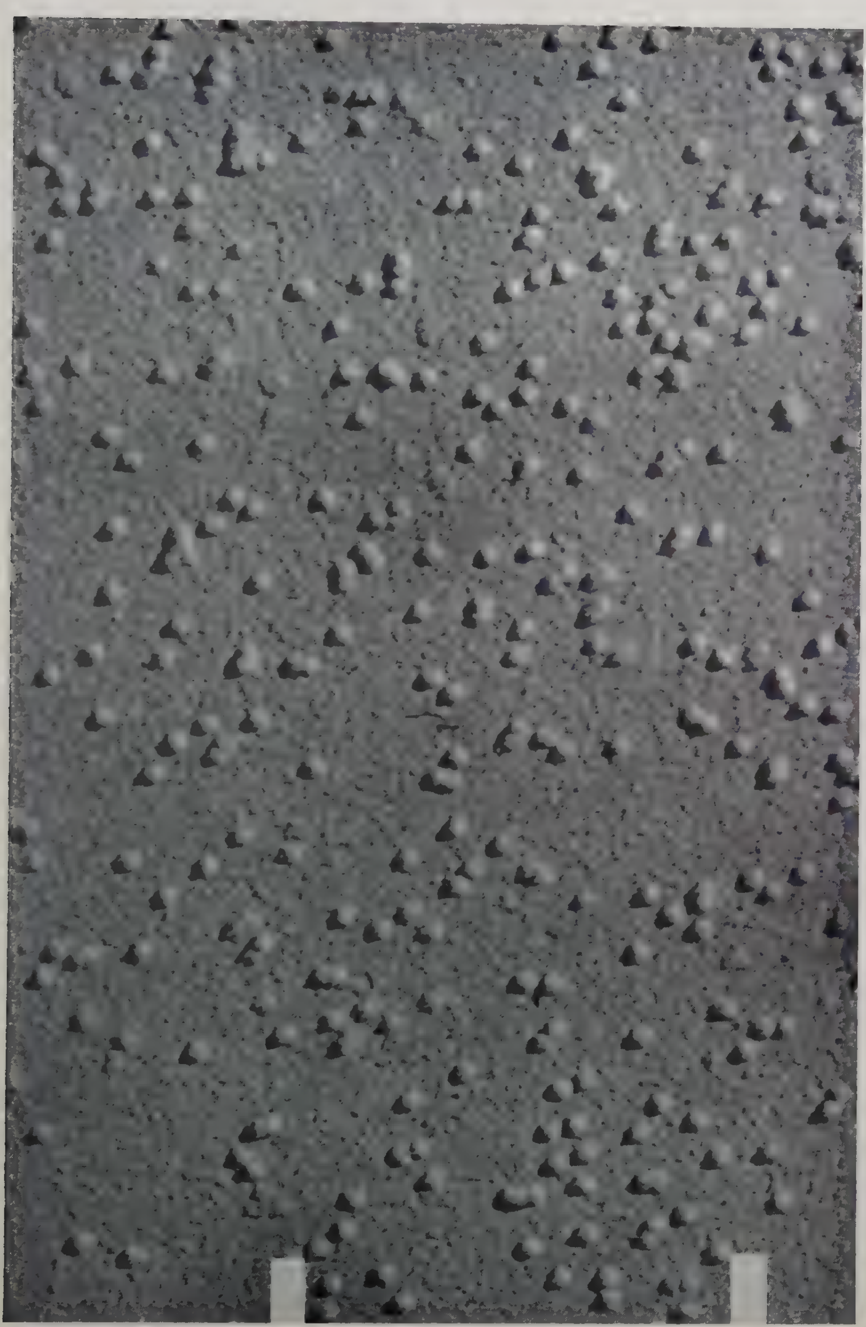

Fig. 12.
Carnation Yellow mosaic virus.

Purified 2 × with ammonium sulfate — dialysed one night. Shadowcast Palladium. Enlargement 55500 ×. Photograph 6.12.'50 (E.f.113).

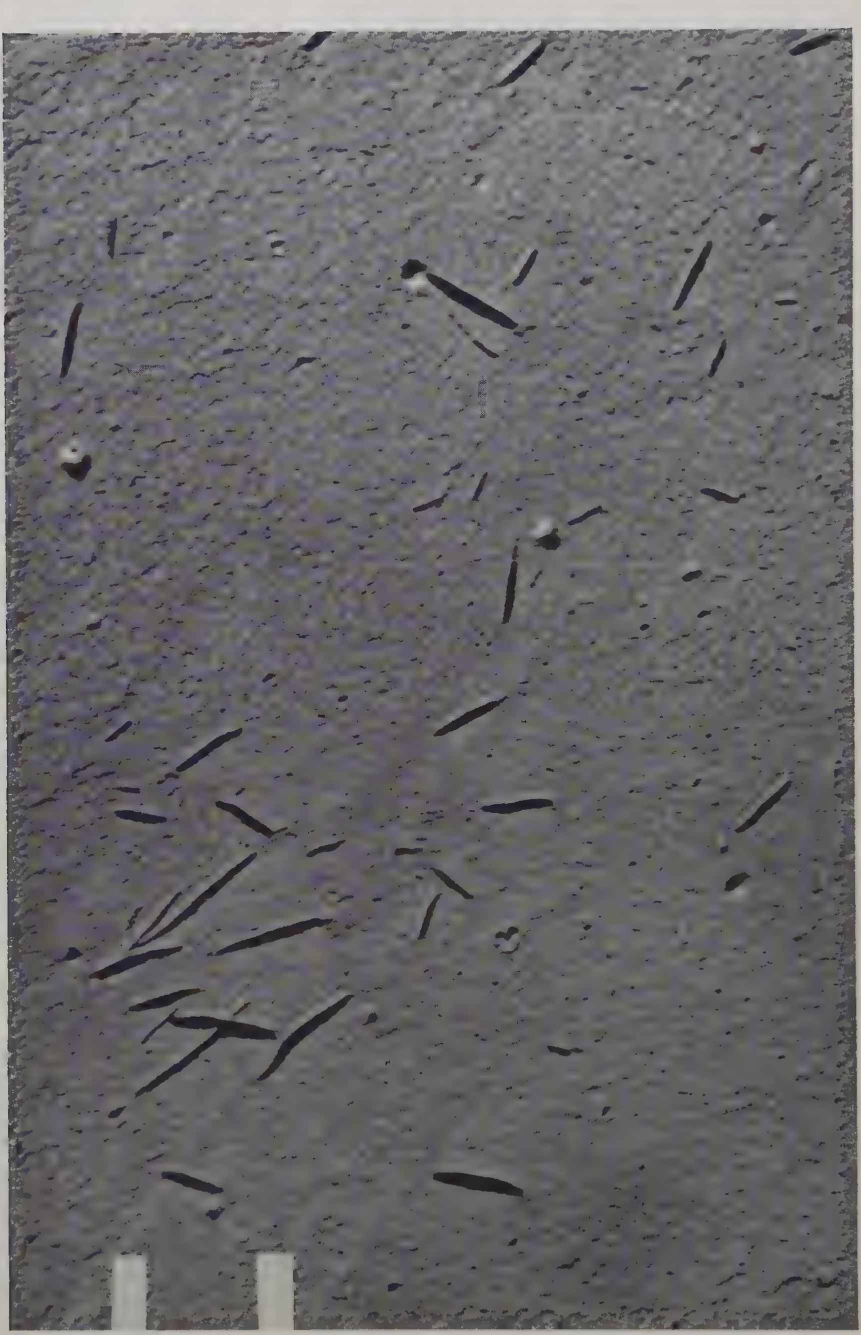

Fig. 13.

Tobacco mosaic virus from tomatoes, purified. Negative serological reaction with normal-serum.

Shadowcast Palladium.
Enlargement 14000 ×. Photograph 20.11.'50 (E.f.72).

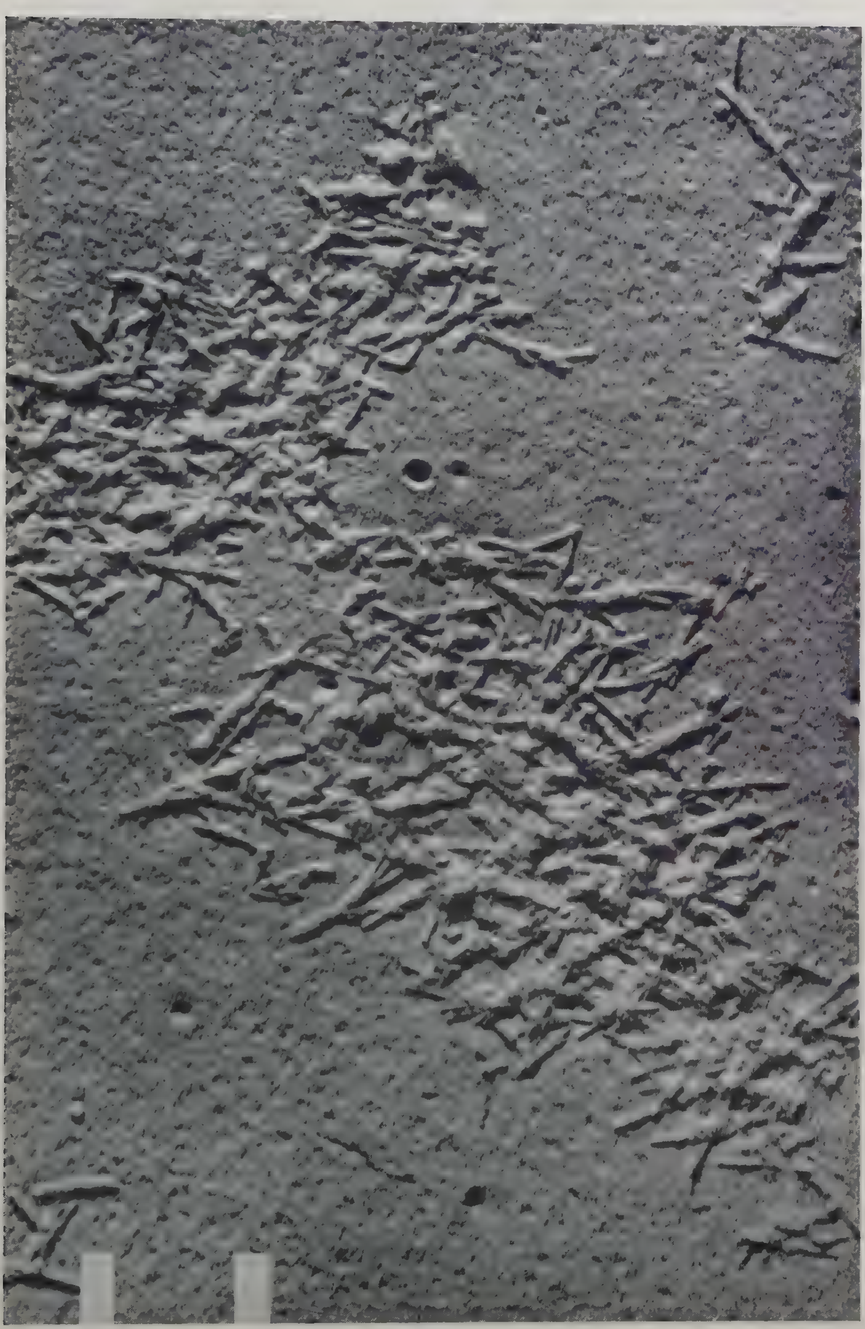

Fig. 14.

Tobacco mosaic virus from tomatoes, purified. Positive serological reaction with tobacco mosaic antiserum.

Shadowcast Palladium.
Enlargement 15000 ×. Photograph 20.10.'50 (E.f.70).

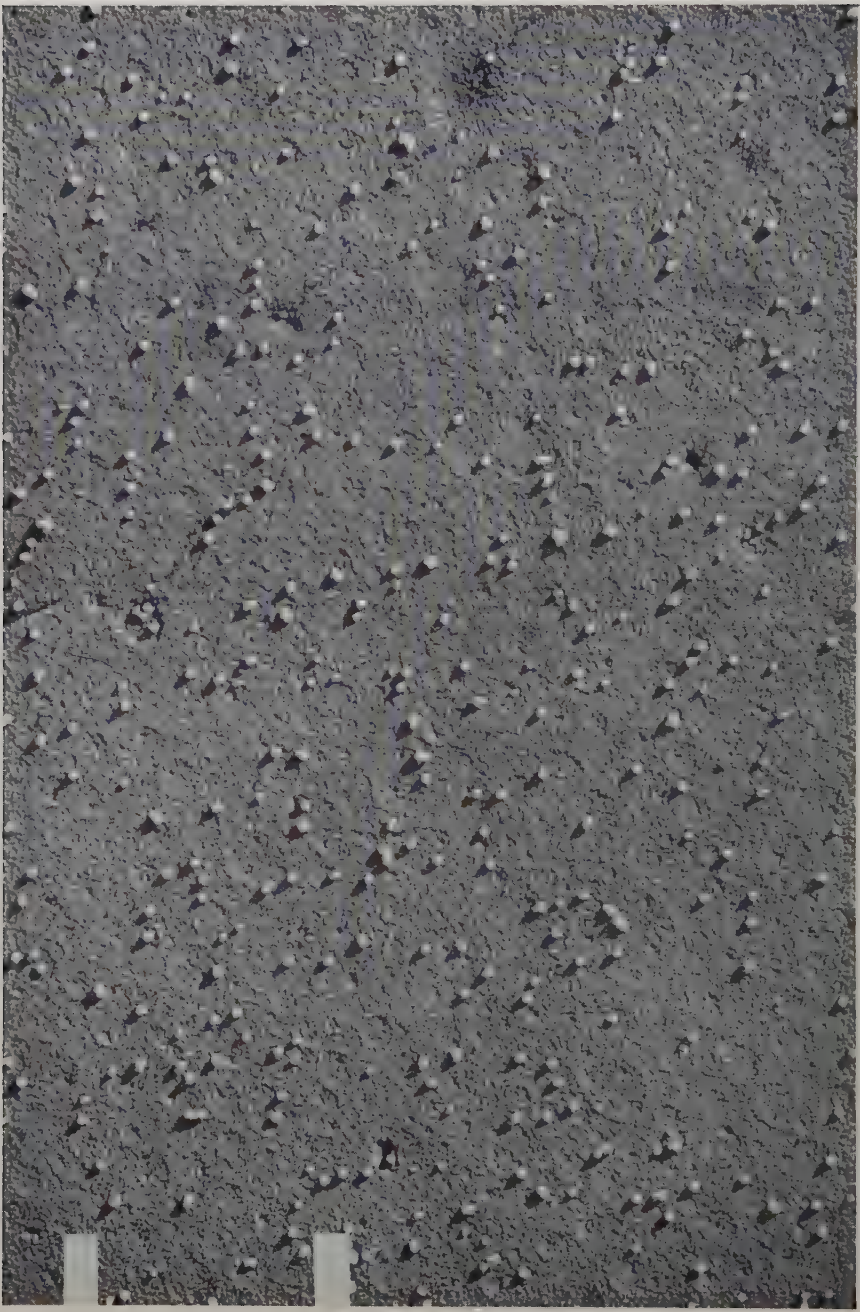

Fig. 15.
Tobacco necrosis virus (Augusta-ziek of tulips) on tobacco plants, foliages minced.

Frozen for a night — centrif. 20 min. at 3500 rot . — purified with ammonium sulfate — centrif. 30 min. at 3500 rot. — 5 × concentrated by centrif. 40 min. at 40.000 rot. — dialysed one night.
Shadowcast Palladium.
Enlargement 28000 ×. Photograph 2.11.'50 (E.f.83).

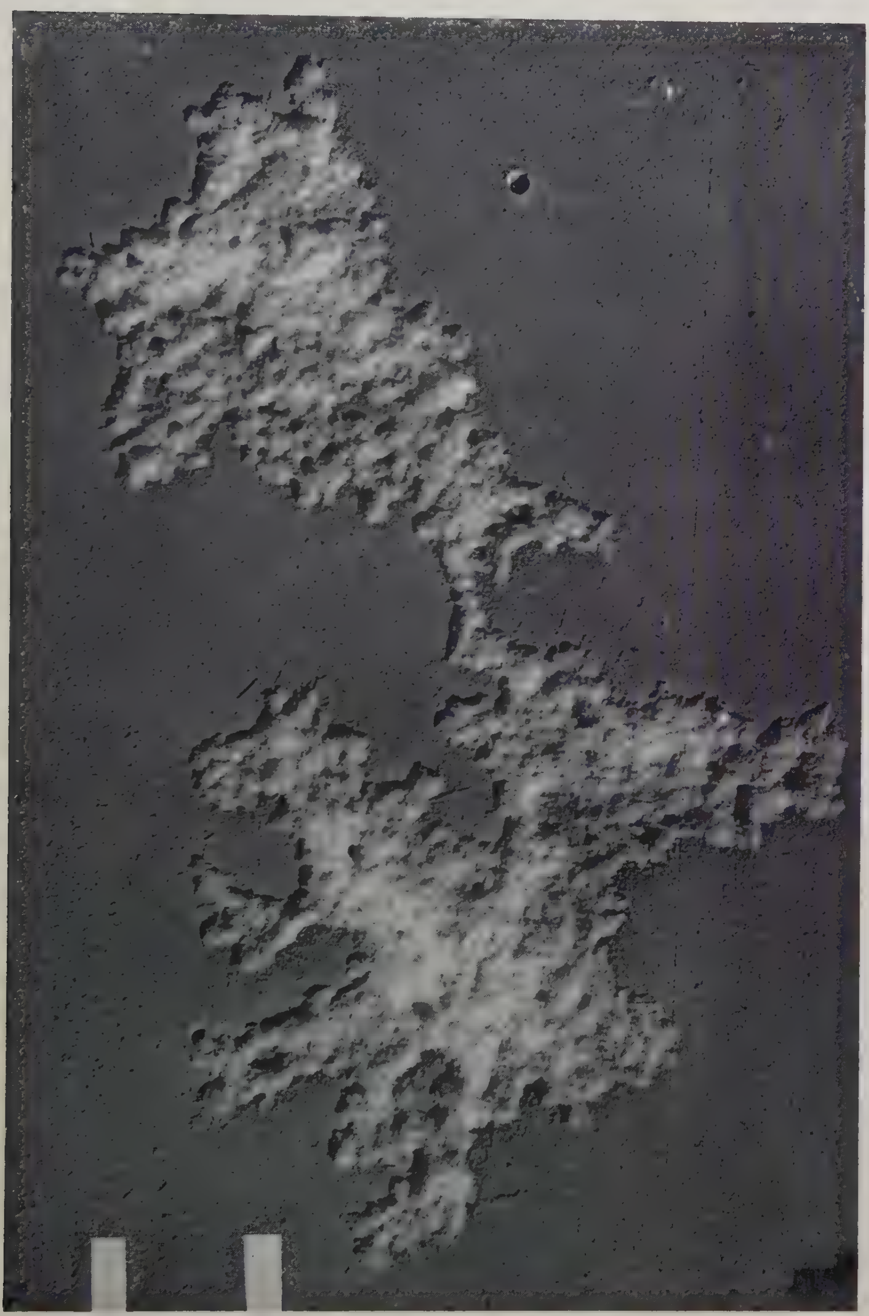

Fig. 16.
Tobacco necrosis virus.
Positive serological reaction with tobacco necrosis-antiserum.

Shadowcast Palladium.
Enlargement 15000 ×. Photograph 2.12.'50 (E.f.109).

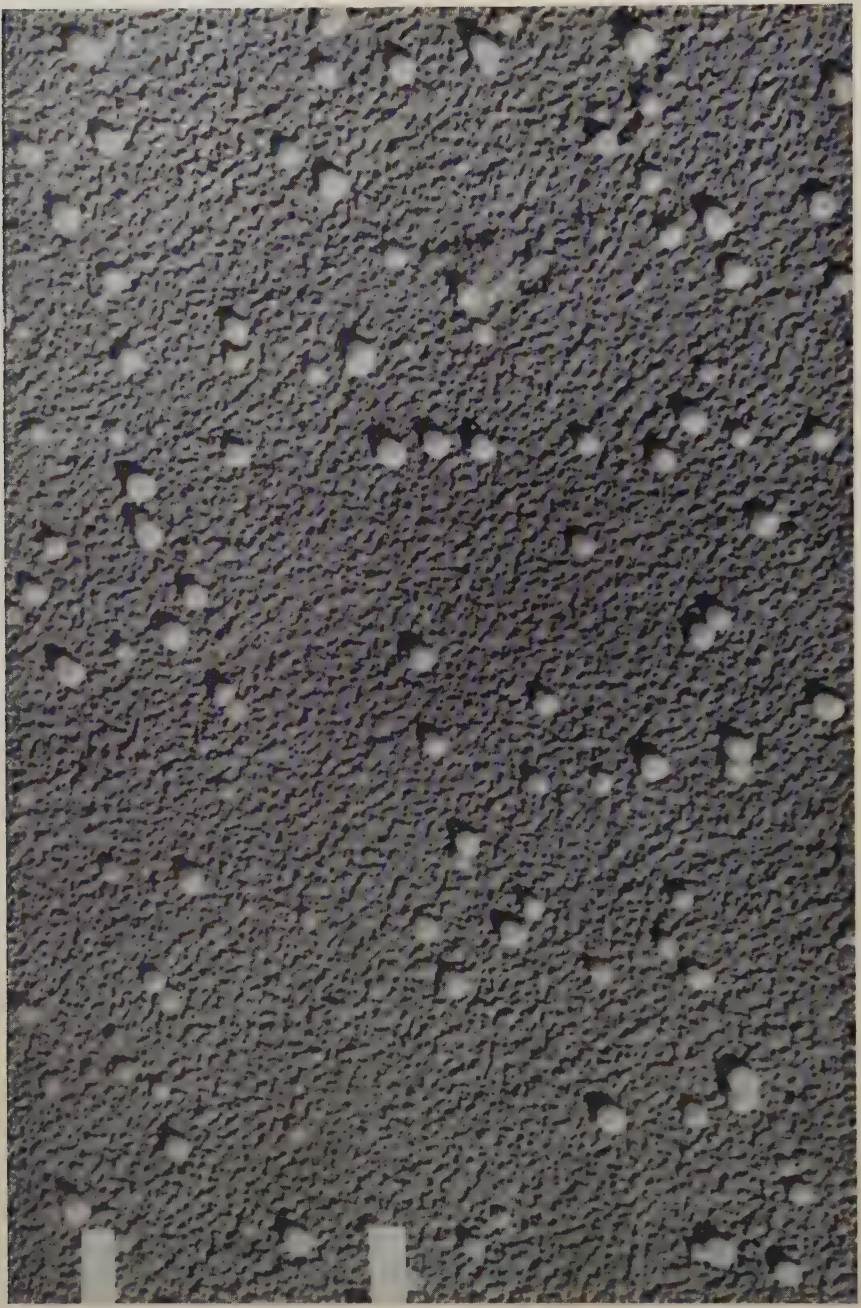

Fig. 17.

Cucumber virus? Transmitted to tobacco, upon which large discolorations developed.

Cut in triple mill — centrif. 20 min. at 8000 rot. — ammonium sulfate — centrif. 20 min. at 8000 rot. — dialysed in water one night.
Shadowcast Palladium.
Enlargement 33000 ×. Photograph 29.11.'50 (E.f.104).

(Aus dem Institut für landw. Bakteriologie und Gärungsbiologie, Eidg. Techn. Hochschule, Zürich).

# UNTERSUCHUNGEN ÜBER DIE PHYSIOLOGIE DER WEINHEFEN I. MITTEILUNG.

## ZUR KENNTNIS DER WACHSTUMSBEDINGUNGEN EINER AUXO-AUTOTROPHEN, SCHWEIZERISCHEN KULTURWEINHEFE

von

**T. WIKÉN** und **O. RICHARD**

(Eingegangen am 8. Februar 1951).

Durch die Untersuchungen von COPPING (4) wurde festgestellt, dass verschiedene Hefearten in Bezug auf den Bedarf an Wuchsstoffen für das Wachstum in synthetischen Nährlösungen, welche neben Zucker nur anorganische Nährsalze enthalten, grosse Unterschiede aufweisen. Während seit dieser Arbeit eine sehr umfangreiche Literatur über die Bedeutung der Vitamine und Aminosäuren für zahlreiche Rassen der Brauerei-, Brennerei- und Presshefen (Bäckerhefen) entstanden ist, war der Einfluss dieser Stoffe auf die Kulturweinhefen bloss Gegenstand weniger Untersuchungen (vgl. SCHOPFER (22), MENZINSKY (15)). In den Lehrbüchern werden bekanntlich jene Hefen zur Art *Saccharomyces cerevisiae* Hansen und diese zur Art *Saccharomyces ellipsoideus* Hansen oder zur Varietät *Saccharomyces cerevisiae* Hansen var. *ellipsoideus* (Hansen) Dekker gezählt (KLÖCKER (12), JÖRGENSEN (9), HENNEBERG (8), KOSSOWICZ (13), GUILLIERMOND (6), GUILLIERMOND und TANNER (7), PRESCOTT und DUNN (20), JÖRGENSEN, HANSEN und LUND (10), JÖRGENSEN und HANSEN (11), BENVEGNIN, CAPT und PIGUET (2) etc.). Aus einigen der erwähnten Lehrbücher sowie aus der Speziallitteratur (OSTERWALDER (17, 18), STELLING-DEKKER (21), etc.) geht hervor, dass die Systematik der ,,Kulturhefen'' bei weitem nicht aufgeklärt ist.

Bei kräftiger Impfung zeigte die von COPPING (4) untersuchte

Rasse von *Saccharomyces ellipsoideus* „slow growth in 3 days" in der Nährlösung nach Reader (Glucose-Mineralsalzlösung mit $(NH_4)_2SO_4$ als Stickstoffquelle). Bei Verwendung sehr kleiner Impfmengen wurde „little growth in 72—96 hours" festgestellt. Ein Zusatz von „Bios" (einerseits autoklavierter Hefeextrakt „Marmite", anderseits zentrifugiertes und autoklaviertes Substrat von Kulturen der auxoautotrophen Hefe *Willia anomala* Hansen (*Hansenula anomala* (Hansen) Sydow) hatte in der Nährlösung von Reader eine fördernde Wirkung auf das Wachstum der Hefe.

Burkholder (3) untersuchte 38 verschiedene Hefen auf ihren Bedarf an Aneurin, Lactoflavin, Pantothensäure, Niacin, Biotin, Inosit und Pyridoxin für das Wachstum in einer synthetischen Nährlösung, welche nebst Glucose und Mineralsalzen Asparagin enthielt. Unter diesen befanden sich auch Stämme der folgenden Hefen: *Saccharomyces cerevisiae* Hansen var. *ellipsoideus* (Hansen) Dekker, *S. chodati* Steiner, *S. globosus* Osterwalder, *S. oviformis* Osterwalder und *S. tubiformis* Osterwalder. Es sei erwähnt, dass der von Burkholder untersuchte Stamm von *S. oviformis* in Uebereinstimmung mit Osterwalders Beschreibung dieser Art imstande war, Galactose zu vergären, während die Rasse, welche dem „Centraalbureau voor Schimmelcultures" in Baarn im Jahre 1923 von Osterwalder zur Verfügung gestellt wurde, nach Stelling-Dekkers Beobachtungen dieses Vermögen nicht besass (18,21). In allen Fällen handelt es sich um Hefen, welche von der Oberfläche reifer Trauben oder aus gärendem Obst- bzw. Traubensaft isoliert worden waren.

In Burkholders Untersuchungen wiesen sämtliche der erwähnten Hefen Biotinheterotrophie auf. Vier Hefearten und zwar *S. cerevisiae* var. *ellipsoideus*, *S. oviformis*, *S. chodati* und *S. tubiformis* erwiesen sich als heterotroph in Bezug auf Pantothensäure. In Abwesenheit von Pyridoxin zeigten *S. oviformis* und *S. chodati* sehr schlechtes Wachstum, während in Abwesenheit des Aneurins *S. globosus* sehr schlechte und *S. cerevisiae* var. *ellipsoideus* sowie *S. oviformis* mittelmässige Vermehrung aufwiesen. Niacin schien nur bei *S. globosus* und Inosit nur bei *S. cerevisiae* var. *ellipsoideus* einen fördernden Einfluss auf das Wachstum auszuüben, während sich Lactoflavin als durchaus inaktiv herausstellte.

Die Hypothese ist aufgestellt worden, dass die wilden Hefen imstande sind, eine synthetische Nährlösung zu assimilieren, welche nur Zucker und anorganische Nährsalze enthält, während den Kulturhefen dieses Vermögen fehlen sollte. Ein Zusatz eines oder einiger Wuchsstoffe scheint für das Wachstum der Kulturhefen notwendig zu sein (vgl. SCHOPFER (22), S. 126, FRIES (5), S. 1).

Im hiesigen Institut sind seit einiger Zeit Untersuchungen über die Physiologie der schweizerischen Kulturweinhefen im Gange. Dabei ergab sich die Notwendigkeit, den Bedarf dieser Hefen an Wuchsstoffen für das Wachstum in genau definierten, synthetischen Substraten kennen zu lernen. Die vorliegende Mitteilung ist ein Beitrag zur Kenntnis der Ernährungsphysiologie der sogenannten ,,Fendant"-Hefe [1]). Diese ist nach LÜTHI (14) eine gegen Alkohol widerstandsfähige Kaltgärhefe (vgl. OSTERWALDER (19)), welche noch bei 4—5° C. einen Traubensaft durchzugären vermag und ihre Tätigkeit erst bei 15—17 Vol.-% Alkohol einstellt. In der Praxis wird sie bei der Bereitung von Weisswein mit hohem Alkoholgehalt, sowie zur Umgärung von Obst- und Traubenweinen verwendet.

In Gärkölbchen nach Einhorn greift die ,,Fendant"-Hefe Glucose, Fructose, Saccharose, Maltose, Raffinose und Galactose unter Entwicklung von Kohlendioxyd an, wobei die Geschwindigkeit der Gasentwicklung in der genannten Reihenfolge abnimmt. Nach der Kohlendioxydmenge zu schliessen, wird Raffinose in Fructose und Melibiose gespalten, wobei die letzte Zuckerart nicht weiter angegriffen wird. Lactose wird nicht vergoren.

In Kulturen auf Gipsblöcken nach ENGEL, welche mit dest. Wasser durchfeuchtet sind, bildet die ,,Fendant"-Hefe bei 20° C. 1—4 Sporen pro Zelle (Abb. 1).

Die Form der ,,Fendant"-Zellen in ungehopfter Bierwürze (8° Bllg) und Süsswein (Verd. 1 : 1) sowie in den Substraten A und B$^1$ geht aus Abb. 2 hervor.

## METHODIK.

Als Kulturgefässe dienten bei sämtlichen Versuchen starkwandige Reagenzgläser ohne Rand von 160 mm Länge und 16 mm lichter Weite. Diese wurden vor jedem Versuch mit heisser Sodalösung gewaschen, dann gründlich mit heissem Leitungswasser und dest. Wasser gespült, hierauf mit dest. Wasser im Autoklaven bei 120° C. während 1 Viertelstunde erhitzt und zuletzt mit dest. Wasser nochmals gespült. Nach Trocknen wurden die Reagenzgläser mit Pfropfen aus chemisch reiner Baumwolle verschlossen und bei 160° C. während 1 Stunde im Trockensterilisator erhitzt. Jedes Kulturröhrchen enthielt bei sämtlichen Versuchen 8 ml Nährlösung. Zur Verwendung kamen folgende Nährsubstrate:

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[1]) Die verwendete Heferasse wurde dem hiesigen Institut von der Eidg. Versuchsanstalt für Obst-, Wein- und Gartenbau, Wädenswil, zur Verfügung gestellt. Herrn Professor Dr F. KOBEL sowie Herrn Dr H. LÜTHI möchten wir an dieser Stelle für ihre Liebenswürdigkeit herzlich danken.

Bierwürzeagar.

Ungehopfte Brauereiwürze wurde im Autoklaven bei 120° C. während 15 Min. erhitzt, nach Abkühlen filtriert und mit dest. Wasser auf etwa 8° Bllg verdünnt, sodann mit 2% Agar versetzt und mit Natronlauge auf pH 5,3—5,5 eingestellt. Nach Abfüllen wurde das Substrat dreimal je 20 Min. im Dampftopf erhitzt.

Nährlösung A (vgl. Modess (16), S. 16).

| | |
|---|---|
| Glucose | 20,0 g |
| Malzextrakt „Biomalz" | 5,0 g |
| $NH_4Cl$ | 0,5 g |
| $KH_2PO_4$ | 0,5 g |
| $MgSO_4.7H_2O$ | 0,5 g |
| $FeCl_3$-Lösung, 1 mg $FeCl_3$/ml | 1,0 ml |
| Dest. Wasser | ad 1000,0 ml |

Die Nährlösung wurde während 20 Min. im strömenden Dampf erhitzt, nach Abkühlen zweimal durch dasselbe Filtrierpapier filtriert, durch Zusatz von Natronlauge auf pH 4, 8—5, 0 eingestellt und sodann bei 120° C. während 15 Min. sterilisiert.

Nährlösung B (vgl. Williams u. Mitarb. (23), S. 86).

| | |
|---|---|
| Glucose | 50,0 g |
| $(NH_4)_2SO_4$ | 6,0 g |
| $KH_2PO_4$ | 2,0 g |
| $MgSO_4.7H_2O$ | 0,25 g |
| $CaCl_2$ | 0,25 g |
| $H_3BO_3$-Lösung, 1 mg $H_3BO_3$/ml | 1,0 ml |
| $ZnSO_4$-Lösung, 1 mg $ZnSO_4$/ml | 1,0 ml |
| $MnCl_2$-Lösung, 1 mg $MnCl_2$/ml | 1,0 ml |
| $Tl_2SO_4$-Lösung, 1 mg $Tl_2SO_4$/ml | 1,0 ml |
| $FeCl_3$-Lösung, 0,5 mg $FeCl_3$/ml | 1,0 ml |
| $CuSO_4$-Lösung, 0,1 mg $CuSO_4$/ml | 1,0 ml |
| KJ-Lösung, 0,1 mg KJ/ml | 1,0 ml |
| Dest. Wasser | ad 1000,0 ml |

Die Nährlösung wurde im Autoklaven 15 Min. bei 120° C. sterilisiert, sodann nach Abkühlen, Einstellen des pH-Wertes auf 4,8—5,0 mit Natronlauge und Zusetzen der Wuchsstoffe im Dampftopf 20 Min. erhitzt.

### Nährlösung $B^1$.

Diese Nährlösung hat dieselbe Zusammensetzung wie Nährlösung B, die Glucoselösung und die Lösung der Mineralsalze wurden aber separat autoklaviert. Nach Mischen und Zusetzen der Wuchsstoffe wurde die Nährlösung im strömenden Dampf während 20 Min. erhitzt. Das so erhaltene Substrat ist praktisch ungefärbt, während die Nährlösung B durch eine bräunlich-gelbe Farbe gekennzeichnet ist.

Aus Tabelle 1 geht hervor, dass Nährlösung $B^1$ für die Vermehrung der ,,Fendant''-Hefe günstiger als Nährlösung B ist und zwar bei Verwendung von Impfmaterial, das in Nährlösung B gewachsen ist. Die Durchlässigkeitswerte der $B^1$-Kulturen sind in den drei Versuchen durchwegs kleiner als die entsprechenden Werte der B-Kulturen (vgl. S. 219).

TABELLE 1.

| Zeit Stunden | I. Impfung: 4.5.50 | | II. Impfung: 20.5.50 | | III. Impfung: 9.6.50 | |
|---|---|---|---|---|---|---|
| | B | $B^1$ | B | $B^1$ | B | $B^1$ |
| 24 | 86,1 | 81,0 | 86,8 | 84,4 | 85,3 | 85,0 |
| 48 | 52,4 | 33,3 | 55,0 | 43,9 | 43,7 | 33,6 |
| 72 | 23,5 | 14,9 | 32,0 | 29,0 | 31,1 | 24,8 |
| 96 | 18,3 | 13,9 | 24,6 | 21,3 | 24,6 | 17,3 |
| 120 | — | — | 21,7 | 16,3 | — | — |
| 144 | 14,9 | 13,8 | — | — | 15,8 | 14,8 |
| 168 | — | — | 15,0 | 14,2 | — | — |
| 192 | 14,2 | 12,7 | — | — | — | — |

Impfkulturen: Nährlösung B, in den Versuchen I und III 48 Stunden alt, in Versuch II 72 Stunden alt. In sämtlichen Versuchen 5 Parallelen.

### Nährlösung C (vgl. Atkin u. Mitarb. (1), S. 98).

| | |
|---|---|
| Glucose | 50,0 g |
| $KH_2PO_4$ | 0,55 g |
| KCl | 0,425 g |

| | |
|---|---|
| $CaCl_2 . 2H_2O$ | 0,125 g |
| $MgSO_4 . 7H_2O$ | 0,125 g |
| $FeCl_3 . 6H_2O$ | 2,5 mg |
| $MnSO_4 . 4H_2O$ | 2,5 mg |
| Kaliumcitrat-Puffer, 0,4 m, pH = 4,8-5,0 | 50,0 ml |
| Caseinhydrolysat, vitaminfrei, 10%, pH = 4,8-5,0 | 40,0 ml |
| Dest. Wasser | ad 1000,0 ml |

Das Caseinhydrolysat gewannen wir aus vitaminfreiem Casein durch Erhitzen mit Salzsäure nach der von Williams und Mitarb. (23), S. 82, beschriebenen Methode. Die Nährlösung wurde während 15 Min. bei 120° C. autoklaviert, sodann nach Abkühlen und Zusetzen der Wuchsstoffe im Dampftopf während 20 Min. erhitzt.

Zur Herstellung der Nährlösungen wurden analysenreine Chemikalien der Firmen Merck (Darmstadt), British Drug Houses Ltd. und Union Chimique Belge benutzt. Die Glucose, das vitaminfreie Casein sowie die Wuchsstoffe (+)-Biotin und meso-Inosit bezogen wir von der Firma Hoffmann-La Roche (Basel).

Die Stammkultur der ,,Fendant''-Hefe wird in üblicher Weise in 10 % Saccharoselösung, mit Leitungswasser hergestellt, aufbewahrt.

Zur Gewinnung des Impfmaterials dienten in den ersten Versuchen 48-72 Stunden alte Kulturen auf Bierwürzeschrägagar. Die Hefezellen wurden mit sterilem dest. Wasser abgespült, abzentrifugiert, in einer neuen Portion dest. Wasser suspendiert und wieder abzentrifugiert. Die gewaschene Zellmasse wurde zuletzt in einer angemessenen Menge sterilem dest. Wasser aufgeschwemmt. Von dieser Zellsuspension wurde 0,1 ml zur Impfung jedes Kulturröhrchens (8 ml Nährlösung) verwendet.

Nachdem festgestellt worden war, dass sich die ,,Fendant''-Hefe in Nährlösung A (mit Malzextrakt), sowie in den synthetischen Substraten B und B$^1$ ohne Zusatz von Wuchsstoffen weiterzüchten lässt, benützten wir diese Nährsubstrate zur Gewinnung der Impfkulturen. Während 8 Monaten sind etwa 120 aufeinanderfolgende Passagen in den erwähnten Nährlösungen mit gutem Erfolg durchgeführt worden. Das Impfmaterial wurde aus 24—96 Stunden alten Kulturen durch Abzentrifugieren und Waschen der Zellen mit sterilem dest. Wasser hergestellt.

Die Bebrütung erfolgte in sämtlichen Versuchen bei 20° C. Dabei schüttelten wir die Kulturen einmal täglich gründlich, so dass die Hefezellen bzw. Zellverbände in der Nährlösung gleichmässig verteilt wurden.

Als Mass des Wachstums wird vorläufig das Absorptionsvermögen der Kulturen für Licht der Wellenlänge 660 m$\mu$ verwendet. Die Durchlässigkeit der zwecks Aufschwemmung der Hefe kräftig geschüttelten Kulturen wurde mit Hilfe eines Beckman-Quarzspektrophotometers in Corex-Küvetten mit einer Schichtdicke von 10 mm gemessen. Die Spaltweite war 0,06 mm. Als Vergleichslösung (Durchlässigkeit = 100%) diente die betreffende nicht geimpfte Nährlösung. Die Abhängigkeit der Lichtschwächung bei der erwähnten Wellenlänge von der Konzentration der Hefesuspensionen wird in einer künftigen Publikation näher erörtert werden. Unmittelbar nach der Impfung schwankte die Durchlässigkeit der Kulturen in den verschiedenen Versuchen zwischen 97,5 und 99,5%.

In den Tabellen 1—10 sind die erwähnten Durchlässigkeitswerte (%) als Mass des Wachstums angegeben.

## Ergebnisse und Diskussion.

Wie bereits erwähnt, wurden in den ersten Versuchen gewaschene Zellen von Bierwürzeschrägagarkulturen als Impfmaterial verwendet. Aus den Tabellen 2 und 3 geht hervor, dass sich diese Zellen in Nährlösung C, d.h. mit Caseinhydrolysat als Stickstoff-

TABELLE 2.

| | Zeit Stunden | Kein Wuchsstoffzusatz | (+)-Biotin 25$\gamma$/1000 ml | meso-Inosit 25 mg/1000 ml |
|---|---|---|---|---|
| I. Impfung: 7.12.49 | 24 | 92,2 | 91,0 | 86,0 |
| | 46 | 67,0 | 70,0 | 61,0 |
| | 70 | 35,1 | 34,8 | 19,5 |
| | 118 | 16,7 | 10,6 | 6,9 |
| | 146 | 15,5 | 10,3 | 6,6 |
| | 166 | — | 12,1 | 5,7 |
| | 190 | 15,1 | 10,3 | 6,0 |
| II. Impfung: 7.2.50 | 18 | 96,1 | 98,0 | 96,8 |
| | 42 | 71,8 | 71,6 | 66,0 |
| | 68 | 39,2 | 39,5 | 21,7 |
| | 92 | 21,0 | 19,2 | 13,2 |
| | 140 | 15,3 | 14,2 | 10,9 |
| | 188 | 12,4 | 10,8 | 8,6 |
| | 312 | 10,5 | 8,7 | 7,3 |

Impfkulturen: Bierwürzeschrägagar, 48 Stunden alt.
Nährlösung: C.
In Versuch I Einzelwerte, in Versuch II 3 Parallelen.

TABELLE 3.

| Zeit Stunden | Kein Wuchsstoffzusatz | (+)-Biotin $25\gamma/1000$ ml | (+)-Biotin $25\gamma/1000$ml meso-Inosit 25mg/1000 ml |
|---|---|---|---|
| 24 | 92,5 | 92,3 | 87,0 |
| 48 | 67,2 | 69,8 | 47,8 |
| 77 | 29,6 | 32,1 | 12,7 |
| 96 | 19,3 | 18,0 | 7,3 |
| 120 | 12,4 | 11,2 | 6,6 |
| 144 | 11,6 | 10,5 | 5,6 |
| 162 | 11,9 | 10,7 | 5,7 |

Impfkultur: Bierwürzeschrägagar, 48 Stunden alt.
Nährlösung: C.
Impfung: 17.12.49. Einzelwerte.

quelle und Glucose als Kohlenstoff- und Energiequelle, ohne irgendwelchen Zusatz von Wuchsstoffen längs einer normalen S-Kurve glatt vermehren. Das (+)-Biotin in einer Konzentration von $25\gamma/1000$ ml übt unter den gegebenen Bedingungen in der Phase der schnellen Zellvermehrung keinen Einfluss aus. Dagegen ist eine schwache bis deutliche, günstige Wirkung des (+)-Biotins auf die Höchstausbeute an Hefezellen bemerkbar. In Bezug auf meso-Inosit kann die Schlussfolgerung gezogen werden, dass dieser Wuchsstoff in einer Menge von 25 mg/1000 ml auf die von Bierwürzeschrägagar stammenden „Fendant"-Zellen sowohl in der Phase der raschen Vermehrung, als auch hinsichtlich der Höchstausbeute eine deutliche, fördernde Wirkung ausübt. Mit einer Kombination von (+)-Biotin und meso-Inosit in den erwähnten Konzentrationen erhält man in Nährlösung C einen günstigen Effekt, welcher von der gleichen Grössenordnung wie derjenige des meso-Inosits allein ist. Die festgestellten, günstigen Wirkungen der Wuchsstoffe meso-Inosit und (+)-Biotin sind als sehr unbedeutend zu bezeichnen.

In späteren Versuchen wurden die zur Impfung verwendeten Hefezellen in Nährlösung A (mit Malzextrakt) gezüchtet. Aus den Tabellen 4 und 5 ist ersichtlich, dass mit diesem Impfmaterial nicht nur in Nährlösung C, welche mit Caseinhydrolysat versetzt ist, sondern auch in Nährlösung B, welche nur Ammoniumsulfat als Stickstoffquelle enthält, eine normale Vermehrung der „Fendant"-Hefe vor sich geht und zwar ohne Zusatz von Wuchsstoffen. Die Verzögerung des Eintretens der schnellen Vermehrung um etwa 24 Stunden in Nährlösung B (Tabelle 4) hängt damit zusammen, dass in diesem Falle eine ausserordentlich kleine Impfmenge zur Verwendung kam (vgl. Tabelle 6). In beiden Substraten hat meso-Inosit in der Konzentration 25 mg/1000 ml eine deutliche, günstige Wirkung auf die Vermehrung der Hefe. Ferner geht aus Tabelle 5 hervor, dass bei Verwendung von Impfmaterial aus Nährlösung A das (+)-Biotin in einer Menge von 25γ/1000 ml der Nährlösung C einen kleinen aber deutlichen, fördernden Einfluss ausübt. Die Wirkung der kombinierten Wuchsstoffe ist gleich gross wie der Effekt des meso-Inosits allein.

TABELLE 4.

| Zeit Stunden | Kein Wuchsstoffzusatz | meso-Inosit 25 mg/1000 ml |
|---|---|---|
| 24 | 98,1 | 98,2 |
| 48 | 95,8 | 94,8 |
| 96 | 73,2 | 37,1 |
| 120 | 37,0 | 17,5 |
| 144 | 22,9 | 10,6 |
| 168 | 20,1 | 9,4 |

Impfkultur: Nährlösung A, 48 Stunden alt.
Nährlösung: B.
Impfung: 13.4.50. 5 Parallelen.

TABELLE 5.

| Zeit Stunden | Kein Wuchsstoffzusatz | (+)-Biotin 25γ/1000 ml | meso-Inosit 25 mg/1000 ml | (+)-Biotin 25γ/1000 ml meso-Inosit 25 mg/1000 ml |
|---|---|---|---|---|
| 24 | 82,6 | 78,9 | 71,7 | 69,2 |
| 48 | 46,3 | 44,5 | 32,5 | 27,6 |
| 72 | 27,4 | 20,2 | 12,1 | 12,1 |
| 96 | 17,6 | 11,6 | 9,2 | 9,6 |
| 120 | 12,3 | 10,7 | 8,3 | 8,4 |

Impfkultur: Nährlösung A, 48 Stunden alt.
Nährlösung: C.
Impfung: 22.5.50. 5 Parallelen.

Zur Herstellung von Impfmaterial der ,,Fendant''-Hefe wurden ferner die rein synthetischen Nährlösungen B und B¹ verwendet. Den in diesen Substraten wachsenden Zellen fehlt die Möglichkeit, aus Bierwürze und Agar bzw. Malzextrakt eventuell unerlässliche Wuchsstoffe aufzunehmen. Die Zellen müssen beim Wachstum in den erwähnten Glucose-Mineralsalzlösungen ihren Wuchsstoffbedarf durch eigene Synthese decken. Wie bereits erwähnt, ist es uns gelungen, mit der ,,Fendant''-Hefe während einer Zeitspanne von 8 Monaten in den Nährlösungen B und B¹ ohne Zusatz von Wuchsstoffen etwa 120 sukzessive Passagen durchzuführen. Es schien nun von Interesse zu sein, die aus einer rein synthetischen Nährlösung stammenden Hefezellen mit denjenigen aus dem mit Malzextrakt versetzten Substrat A zu vergleichen und zwar hinsichtlich der in rein synthetischen Nährlösungen stattfindenden Vermehrung. Aus Tabelle 6 gehen die Ergebnisse hervor, welche bei Impfung der Nährlösung B¹ mit Zellen aus Nährlösung A bzw. B¹ erhalten wurden. Es ist ersichtlich, dass in Bezug auf Vermehrung in synthetischer Nährlösung nur sehr kleine Unterschiede vorliegen zwischen den Impfzellen, welche Gelegenheit hatten, Malzextrakt als Wuchsstoffquelle auszunützen, und denjenigen, welche lediglich auf eigene Wuchsstoffsynthese angewiesen waren. Ferner wird bestätigt, dass meso-Inosit in der Konzentration 25 mg/1000 ml einen fördernden Einfluss auf das Wachstum der ,,Fendant''-Hefe in Anwesenheit von Ammoniumsulfat als einzige Stickstoffquelle ausübt (vgl. Tabelle 4).

TABELLE 6.

| Zeit Stunden | Impfkultur: Nährlösung A | | Impfkultur: Nährlösung B¹ | |
|---|---|---|---|---|
| | Kein Wuchsstoffzusatz | meso-Inosit 25 mg/1000 ml | Kein Wuchsstoffzusatz | meso-Inosit 25 mg/1000 ml |
| 24 | 82,6 | 80,3 | 84,0 | 78,4 |
| 48 | 49,5 | 25,0 | 40,4 | 28,8 |
| 72 | 23,4 | 12,7 | 22,1 | 14,5 |
| 96 | 22,5 | 10,4 | 19,3 | 13,1 |
| 120 | 16,9 | 10,4 | 14,8 | 10,3 |
| 168 | 15,5 | 10,0 | 14,0 | 10,5 |

Nährlösung: B¹.
Impfung: 27.4.50. In beiden Versuchen 5 Parallelen.

Versuche wurden sodann über den Einfluss des (+)-Biotins und des meso-Inosits sowie der Kombination dieser Wuchsstoffe auf die Vermehrung der aus den Nährlösungen B und $B^1$ stammenden ,,Fendant''-Zellen in den nämlichen Substraten durchgeführt. Aus Tabelle 7 können die in zwei typischen Versuchen gewonnenen Ergebnisse ersehen werden.

TABELLE 7.

| Zeit Stunden | Kein Wuchsstoff-zusatz | (+)-Biotin 25γ/1000 ml | meso-Inosit 25 mg/1000 ml | (+)-Biotin 25γ/1000 ml meso-Inosit 25 mg/1000 ml |
|---|---|---|---|---|
| I. Impfkultur: Nährlösung B, 96 Stunden alt; Nährlösung: B; Impfung: 17.5.50 | | | | |
| 24 | 82,3 | 80,2 | 77,7 | 73,0 |
| 48 | 51,3 | 44,9 | 41,3 | 42,5 |
| 72 | 37,9 | 27,2 | 22,5 | 22,5 |
| 96 | 25,5 | 19,1 | 14,8 | 10,9 |
| 120 | 23,1 | 15,0 | 12,2 | 10,6 |
| 168 | 16,7 | 12,3 | 10,9 | 10,1 |
| II. Impfkultur: Nährlösung $B^1$, 96 Stunden alt; Nährlösung: $B^1$; Impfung: 15.5.50. | | | | |
| 24 | 81,6 | 76,4 | 68,9 | 70,8 |
| 48 | 51,1 | 41,4 | 34,6 | 27,3 |
| 72 | 24,5 | 18,9 | 15,5 | 13,0 |
| 96 | 17,0 | 14,8 | 11,3 | 10,3 |
| 120 | 17,6 | 14,0 | 12,7 | 9,9 |
| 168 | 13,8 | 12,2 | 10,2 | 9,0 |

In beiden Versuchen 5 Parallelen.

Wie zu erwarten war, findet in beiden Substraten ohne Zusatz von Wuchsstoffen eine normale Vermehrung der Hefe längs einer S-förmigen Kurve statt. In Gegenwart von 25γ (+)-Biotin/1000 ml Nährlösung ist eine kleine aber deutliche, günstige Wirkung bemerkbar. Mit meso-Inosit in einer Menge von 25 mg/1000 ml Substrat wird wie in der Caseinhydrolysat-haltigen Nährlösung C ein grösserer, günstiger Effekt erhalten. Die Wirkung der Kombination beider Wuchsstoffe ist praktisch gleich wie der Einfluss des meso-Inosits allein. In der bräunlich-gelben Nährlösung B, bei deren Herstellung die Glucose zusammen mit den Mineralsalzen autoklaviert wird, ist das Wachstum der ,,Fendant''-Hefe durchgehends schlechter als in der praktisch ungefärbten Nährlösung $B^1$, bei deren Bereitung Zucker und Mineralsalze separat autoklaviert werden, was eine

TABELLE 8.

| | Zeit Stunden | Kein Wuchsstoffzusatz | γ(+)-Biotin/1000 ml | | | mg meso-Inosit/1000 ml | | |
|---|---|---|---|---|---|---|---|---|
| I. Impfung: 1.6.50 | | | 2,5 | 25 | 250 | 2,5 | 25 | 250 |
| | 24 | 95,9 | 94,0 | 94,2 | 94,6 | 95,2 | 94,7 | 94,7 |
| | 48 | 69,4 | 52,7 | 53,3 | 49,7 | 59,6 | 53,6 | 50,8 |
| | 72 | 28,3 | 16,0 | 15,6 | 15,7 | 18,7 | 17,4 | 15,5 |
| | 96 | 20,0 | 15,9 | 15,0 | 14,9 | 14,1 | 12,2 | 12,7 |
| | 144 | 15,7 | 12,7 | 12,6 | 13,4 | 12,7 | 11,3 | 10,2 |
| II. Impfung: 13.6.50 | | | 0,025 | 0,25 | 2,5 | 0,025 | 0,25 | 2,5 |
| | 24 | 85,4 | 83,1 | 82,9 | 85,8 | 85,1 | 82,4 | 79,0 |
| | 48 | 46,1 | 36,5 | 36,0 | 35,5 | 48,4 | 48,6 | 34,2 |
| | 72 | 26,5 | 19,4 | 16,9 | 15,9 | 25,7 | 25,9 | 19,3 |
| | 96 | 18,6 | 12,0 | 12,0 | 12,5 | 19,9 | 20,0 | 14,1 |
| | 120 | 17,3 | 12,9 | 11,7 | 12,1 | 16,7 | 18,0 | 11,8 |
| III. Impfung: 20.6.50 | | | 0,00025 | 0,0025 | 0,025 | 0,025 | 0,25 | 2,5 |
| | 24 | 81,3 | 77,3 | 77,9 | 75,9 | 76,5 | 75,0 | 70,5 |
| | 48 | 44,1 | 45,4 | 37,6 | 28,7 | 42,8 | 45,1 | 32,2 |
| | 72 | 28,2 | 30,8 | 23,0 | 20,2 | 28,8 | 30,1 | 19,6 |
| | 96 | 19,1 | 21,4 | 15,9 | 11,9 | 20,0 | 20,0 | 13,2 |
| IV. Impfung: 11.7.50 | | | 0,00025 | 0,0025 | 0,025 | 0,25 | 2,5 | 25 |
| | 24 | 98,1 | 97,9 | 97,8 | 97,8 | 97,8 | 97,4 | 97,4 |
| | 48 | 53,1 | 51,1 | 50,0 | 48,4 | 50,5 | 43,3 | 40,3 |
| | 72 | 18,8 | 18,6 | 17,7 | 14,2 | 16,8 | 13,6 | 13,5 |
| | 96 | 11,1 | 11,3 | 10,4 | 9,1 | 11,1 | 7,9 | 8,2 |
| | 144 | 9,0 | 9,3 | 8,8 | 7,5 | 8,5 | 7,6 | 7,3 |
| V. Impfung: 27.6.50 | | | 0,0000025 | 0,000025 | 0,00025 | 0,0025 | 0,025 | 0,25 |
| | 24 | 80,8 | 81,0 | 80,7 | 80,2 | 81,8 | 81,5 | 80,2 |
| | 48 | 47,8 | 46,8 | 46,4 | 47,2 | 49,5 | 46,0 | 47,3 |
| | 72 | 30,5 | 29,9 | 30,0 | 29,0 | 28,8 | 30,5 | 29,0 |
| | 96 | 17,0 | 16,9 | 17,8 | 18,4 | 19,3 | 18,4 | 18,4 |
| | 144 | 16,2 | 17,3 | 16,9 | 17,1 | 17,6 | 17,3 | 17,1 |

Impfkulturen: Nährlösung B[1], in den Versuchen I, II und V 96 Stunden alt, in Versuch III 72 Stunden alt, in Versuch IV 24 Stunden alt.

Nährlösung: B[1].

In den Reihen ohne Wuchsstoffzusatz 10 Parallelen, in übrigen Reihen 5 Parallelen.

Bestätigung der in Tabelle 1 zusammengestellten Ergebnisse darstellt.

Es ist somit bewiesen, dass die Kulturweinhefe ,,Fendant'' auxoautotropher Natur ist, dass aber (+)-Biotin und meso-Inosit auf die Vermehrung der Hefe unter gewissen Bedingungen einen kleinen aber deutlichen, fördernden Einfluss ausüben. In diesem Zusammenhang sei erwähnt, dass die Abwesenheit von Wuchsstoffen in den zur Herstellung der Nährlösungen verwendeten Chemikalien durch die im Institute heute laufenden Untersuchungen über Mikroorganismen auxoheterotropher Natur (*Clostridium-*, *Saccharomyces-*, *Neurospora-*, *Phycomyces-*, *Cenococcum-* und *Marasmius*-Stämme, etc.) einwandfrei festgestellt worden ist.

Schliesslich wurden sowohl in Nährlösung B[1] als in Nährlösung C Versuche durchgeführt, um die kleinste Konzentration des (+)-Biotins bzw. meso-Inosits festzustellen, bei welcher eine wachstumsfördernde Wirkung auftritt. Die Ergebnisse einiger typischen Versuche sind in den Tabellen 8—10 zusammengestellt. Aus diesen

TABELLE 9.

| | Zeit Stunden | Kein Wuchsstoffzusatz | $\gamma$(+)-Biotin/1000 ml | | | mg meso-Inosit/1000 ml | | |
|---|---|---|---|---|---|---|---|---|
| I. Impfung: 9.6.50 | | | 2,5 | 25 | 250 | 2,5 | 25 | 250 |
| | 24 | 84,0 | 82,0 | 82,4 | 81,7 | 71,8 | 72,8 | 71,8 |
| | 48 | 44,6 | 37,5 | 37,8 | 37,1 | 25,6 | 23,7 | 22,6 |
| | 72 | 21,6 | 17,0 | 17,1 | 17,1 | 11,0 | 8,5 | 9,4 |
| | 96 | 11,0 | 9,2 | 9,9 | 9,8 | 7,5 | 6,8 | 7,2 |
| II. Impfung: 27.6.50 | | | 0,000025 | 0,00025 | 0,0025 | 0,025 | 0,25 | 2,5 |
| | 24 | 86,5 | 86,1 | 86,2 | 84,6 | 85,7 | 85,1 | 85,1 |
| | 48 | 61,8 | 62,9 | 62,3 | 61,7 | 61,0 | 63,5 | 60,9 |
| | 72 | 43,2 | 45,1 | 44,0 | 38,7 | 44,5 | 43,8 | 24,9 |
| | 96 | 30,5 | 31,0 | 32,0 | 20,2 | 30,9 | 31,5 | 12,1 |
| | 144 | 16,8 | 16,7 | 17,3 | 12,2 | 16,4 | 16,3 | 8,6 |

Impfkulturen: Nährlösung A, in Versuch I 48 Stunden alt, in Versuch II 72 Stunden alt.
Nährlösung: C.
In den Reihen ohne Wuchsstoffzusatz 10 Parallelen, in übrigen Reihen 5 Parallelen.

TABELLE 10.

| | Zeit Stunden | Kein Wuchsstoffzusatz | γWuchsstoff/1000 ml | | | |
|---|---|---|---|---|---|---|
| I. Impfung: 13.6.50 | | | 0,025 | 0,25 | 2,5 | (+)-Biotin |
| | 24 | 89,9 | 83,9 | 83,4 | 83,2 | |
| | 48 | 57,5 | 50,0 | 47,4 | 44,3 | |
| | 72 | 19,6 | 18,7 | 14,9 | 16,7 | |
| | 96 | 12,1 | 9,4 | 10,8 | 9,4 | |
| II. Impfung 20.6.50 | | | 0,00025 | 0,0025 | 0,025 | |
| | 24 | 90,4 | 89,9 | 86,8 | 85,8 | |
| | 48 | 53,6 | 55,0 | 49,4 | 47,6 | |
| | 72 | 27,5 | 25,0 | 18,6 | 18,3 | |
| | 96 | 15,6 | 15,1 | 12,3 | 11,2 | |
| III. Impfung: 11.7.50 | | | 0,25 | 2,5 | 25 | meso-Inosit |
| | 24 | 99,0 | 98,1 | 98,9 | 98,8 | |
| | 48 | 86,3 | 79,9 | 86,1 | 86,6 | |
| | 72 | 47,9 | 42,0 | 36,6 | 32,8 | |
| | 96 | 20,3 | 17,3 | 12,7 | 11,3 | |
| | 144 | 10,5 | 9,4 | 8,7 | 7,7 | |
| IV. Impfung: 13.6.50 | | | 0,025 | 0,25 | 2,5 | |
| | 24 | 82,3 | 81,4 | 79,1 | 75,4 | |
| | 48 | 48,6 | 49,0 | 46,3 | 36,6 | |
| | 72 | 18,1 | 17,4 | 19,5 | 13,0 | |
| | 96 | 10,3 | 10,0 | 10,4 | 8,5 | |

Impfkulturen: Nährlösung A, in den Versuchen I und IV 96 Stunden alt, in den Versuchen II und III 24 Stunden alt.
Nährlösung: C.
In den Reihen ohne Wuchsstoffzusatz 10 Parallelen, in übrigen Reihen 5 Parallelen.

Zahlen geht hervor, dass in beiden Nährlösungen die betreffenden Mindestkonzentrationen innerhalb der Grenzen 0,0025 — 0,025γ (+)-Biotin/1000 ml bzw. 0,25–2,5 mg meso-Inosit/1000 ml liegen.

Die auxoautotrophe „Fendant"-Hefe reagiert somit glatt auf die Anwesenheit von 0,000025 $\gamma$ (+)-Biotin bzw. 2,5 $\gamma$ meso-Inosit in 1 ml der erwähnten Substrate.

Die Untersuchungen über die Physiologie der Weinhefen werden von der Abteilung für Landwirtschaft des Eidg. Volkwirtschaftsdepartements aus dem Weinbaufonds unterstützt. Fräulein L. BENDEL danken wir bestens für sorgfältige Assistenz.

## Summary.

It is a wide-spread opinion that the wild yeasts are auxo-autotrophic and thus grow in vitamin-free synthetic media, whereas the culture yeasts or tame yeasts in the course of time have become more or less auxo-heterotrophic and are unable to develop in synthetic nutrient solutions unless one or more growth factors are added.

The Fendant yeast is a selected Swiss culture strain used at low temperatures for production of (white) wines of high alcoholic content. In water on sterile gypsum blocks it forms 1 to 4 spores in each cell. In Einhorn saccharimeters it attacks glucose, fructose, sucrose, maltose, raffinose, and galactose with formation of carbon dioxide. The rate of gas production decreases in the sugar series mentioned, the fermentation of glucose and galactose under the experimental conditions being completed after 24 and 96 hours, respectively. Of the raffinose molecule probably only 1/3 is fermented. Lactose is not fermented.

The present paper reports some results obtained in examining the growth of Fendant culture yeast in nutrient solutions containing glucose, mineral salts, and ammonium sulfate or vitamin-free casein hydrolysate as a source of nitrogen. The growth obtained in cultures without addition of growth factors was compared with that observed in the corresponding media containing (+)-biotin and meso-inositol, added singly or in combination. The estimation of yeast growth was based upon the absorption of light in the Beckman photoelectric quartz spectrophotometer at the wave length 660 m$\mu$.

The results show conclusively that the Fendant tame yeast is an auxo-autotrophic strain. It is capable of synthesizing its cellular constituents in a chemically defined medium containing only glucose and mineral salts including ammonium sulfate. A vitamin-

free casein hydrolysate may be substituted for ammonium sulfate as a source of nitrogen.

The addition of (+)-biotin in amounts greater than 0.0025—0.025$\gamma$/1000 ml and meso-inositol in quantities greater than 0.25—2.5 mg/1000 ml results in a very small increase in growth.

During a period of 8 months the Fendant culture yeast has been successfully subcultured about 120 times in the synthetic, vitamin-free nutrient solution containing glucose as a source of carbon and energy and mineral salts including ammonium sulfate as a source of nitrogen.

The theory of the auxo-heterotrophic nature of tame yeasts involves an inadmissible generalization.

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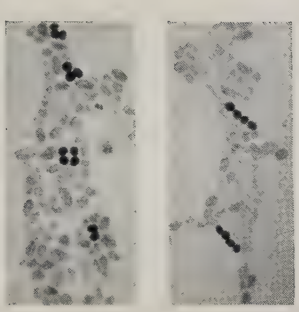

Abb. 1. Sporenbildung der „Fendant"-Zellen in 6 Tage alten Gipsblock-Kulturen. Färbung mit Karbolfuchsin nach Behandlung mit Chromsäure. Vergr. 425 ×.

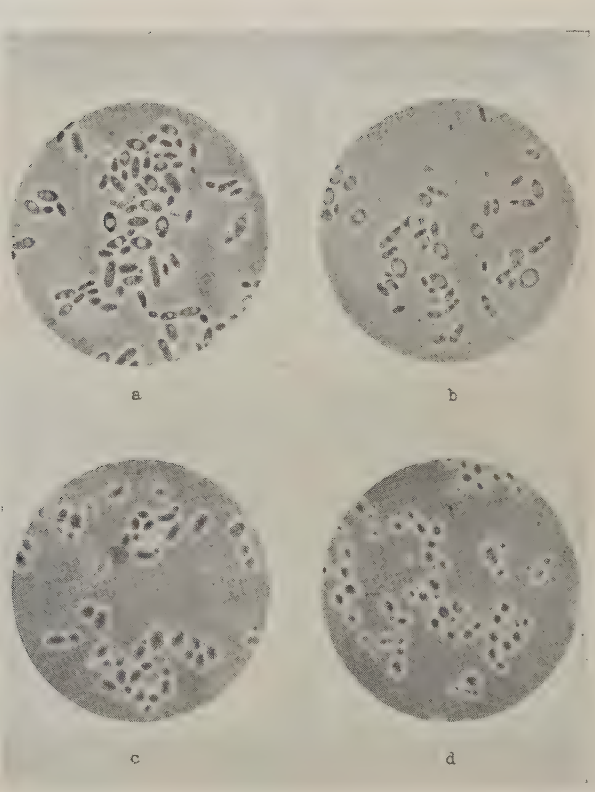

Abb. 2. Aussehen der „Fendant"-Zellen in verschiedenen Nährlösungen. 48 Stunden alte Kulturen. Lebende Zellen. Phasenkontrast. Verg. 300 ×

a. Ungehopfte Bierwürze (8° Bllg).
b. Süsswein (Verd. 1 : 1).
c. Nährlösung A.
d. Nährlösung B′.

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# ORIGINE SPONTANEE DES MUTANTS DE *E. COLI* V PRODUISANT LA COLICINE M

par

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(Reçu le 15 mars 1951).

Nous avons montré antérieurement (5,8) que les mutants, résistant aux bactériophages T.1, T.5 ou T.7, de la souche *E. coli* V de Gratia ont acquis la propriété héréditaire nouvelle de produire, outre la colicine V caractéristique de la souche-mère dont ils dérivent, une deuxième colicine, désignée M, entièrement distincte.

Comme il s'agit d'une mutation spécifique liée à l'action des phages T.1, T.5 ou T.7, la question se pose de savoir si les mutants produisant la colicine M ont une origine spontanée et sont simplement sélectionnés par les phages, ou si la mutation résulte de l'induction spécifique d'une propriété nouvelle.

Par la technique de Luria et Delbrück (10), on démontre aisément que des mutants de *E. coli* V résistant aux phages T.1, T.5 et T.7 existent dans la culture initiale avant l'intervention des phages et ont donc une origine spontanée. Mais rien ne permet d'affirmer que ces mutants résistants, préexistant dans la souche-mère, possèdent déjà la propriété antibiotique nouvelle, puisque l'action sélective des phages est déterminée uniquement par le facteur résistance et non par le caractère antibiotique. Il est fort possible que l'apparition du caractère antibiotique nouveau soit consécutive à une action directe des phages et ne se manifeste évidemment que chez les cellules ayant échappé à la lyse, c'est-à-dire les mutants résistants préexistants.

## 1. Arguments en faveur de l'induction par les phages.

Si le caractère antibiotique nouveau est induit par les phages, on comprend aisément qu'il soit associé à la résistance à ces phages. Mais si la mutation est spontanée, on doit alors admettre qu'elle est génétiquement liée à la résistance aux phages T.1, T.5 et T.7, et on ne voit pas la raison d'un tel lien.

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[1]) Associé du Fonds National de la Recherche Scientifique.

On n'observe jamais l'apparition spontanée du mutant produisant la colicine M. Nous avons étudié une centaine de subcultures de *E. coli* V, issues chacune d'une colonie isolée sur gélose; elles ne produisent aucune la colicine M. Des mutants de *E. coli* V résistant à d'autres phages que T.1, T.5 ou T.7, ne produisent pas non plus la colicine M. D' ailleurs, la souche *E. coli* V, isolée par GRATIA il y a vingt cinq ans et étudiée depuis dans plusieurs laboratoires et notamment par nous-même pendant plusieurs années, n'a jamais présenté qu'un seul type de mutant antibiotique. Ce mutant, observé et décrit par BORDET (1) se caractérise par la production, au lieu de colicine V, d'une colicine de spécificité identique mais s'en distinguant par sa thermolabilité et ses propriétés antigéniques. Le mutant apparaissant sous l'action des phages T.1, T.5 ou T.7 ne lui ressemble en rien puisqu'il produit, en plus de la colicine V, une seconde colicine de spécificité entièrement distincte.

Un dernier argument enfin en faveur de l'induction de la mutation par les phages est la parenté existant entre la colicine M et les phages qui déterminent sa production. Nous avons en effet montré (6, 8) que, chez une souche sensible à la fois à la colicine M et au phage T.1, les mutants résistants sélectionnés par la colicine M résistent aussi au phage T.1 et, inversement, les mutants résistants sélectionnés par ce phage T.1 résistent aussi à la colicine M. Il existe une résistance croisée spécifique à ces deux agents.

Ces arguments nous avaient amené (6, 9) à conclure à l'induction de la mutation par les phages. De nouvelles recherches ont cependant modifié nos conceptions.

## 2. ARGUMENTS EN FAVEUR D'UNE ORIGINE SPONTANÉE.

Les cultures de *E. coli* V déterminent sur la souche sensible coli $\varphi$ la large zone d'inhibition caractéristique de la colicine V mais n'ont pas d'action sur un mutant de $\varphi$ résistant à cette colicine (mutant désigné $\varphi/V$). Certaines subcultures de V cependant montrent encore sur $\varphi/V$ une ébauche d'action qui manque toujours lorsqu'on les fait agir sur le mutant issu de $\varphi/V$, résistant à la colicine M (mutant $\varphi$/VM). Il semble donc que certaines subcultures de V produisent tout au moins des traces de colicine M.

La souche coli V est la seule à donner un mutant antibiotique sous l'action des phages T.1, T.5 et T.7, Nous avons étudié l'action de ces phages sur de nombreuses souches parmi lesquelles certaines sont du type coli V et produisent la colicine V ,d'autres produisent une autre colicine et d'autres enfin sont inactives. Les mutants, résistant aux phages T.1, T.5 ou T.7, dérivés de ces souches, sont identiques à la souche-mère et ne produisent aucun la colicine M. Par contre, la souche coli VA, que nous a aimablement transmise Bordet (1) et qui a été dérivée de la souche coli V de Gratia il y a plus de dix ans, produit, après action des phages T.1, T.5 ou T.7, la colicine M. La propriété de produire la colicine M semble donc dépendre d'un facteur inhérent à la souche coli V.

Les mutants de coli V produisant la colicine M apparaissent indifféremment sous l'action de T.1, de T.5 ou de T.7 et sont simultanément résistant à ces trois phages. Cette résistance croisée n'est cependant pas de règle pour toutes les souches sensibles considérées (7), ce qui prouve donc que ces trois phages ne sont pas identiques. Ils diffèrent d'ailleurs l'un de l'autre morphologiquement et sérologiquement (cf. 2). On conçoit dès lors qu'ils exercent sur coli V une action sélective identique mais non le même effet inducteur.

Nous avons cherché à obtenir les mutants de coli V produisant la colicine M indépendamment de l'action des phages T.1, T.5 ou T.7 en faisant agir d'autres agents sélectifs et notamment différentes colicines. La souche *E. coli* V n'est pas sensible à la colicine V qu'elle produit, mais elle est inhibée par plusieurs autres colicines. Nous avons dérivé des mutants de coli V résistant aux diverses colicines auxquelles cette souche est sensible et nous avons étudié leur action sur la souche indicatrice sensible *E. coli* $\varphi$ et sur un mutant de coli $\varphi$ résistant à la colicine V ($\varphi$/V), suivant notre technique habituelle (3). La plupart de ces mutants dérivés de la souche *E. coli* V déterminent sur *E. coli* $\varphi$ la large zone d'inhibition caractéristique de la colicine V et, comme la souche-mère, n'ont plus d'action sur le mutant $\varphi$/V. Le mutant de *E. coli* V, résistant à la colicine C, se comporte cependant différemment et détermine encore sur $\varphi$/V une zone d'inhibition du type colicine M. Par l'étude de mutants résistants appropriés, il est aisé de constater que la colicine produite par le mutant de coli V résistant à la colicine C est identique à la colicine M que produisent les mutants de coli V résistant aux phages T.1, T.5 ou T.7.

Ainsi donc, un agent très différent des phages T.1, T.5 et T.7, la colicine C, est également capable de déterminer l'apparition du mutant de coli V produisant la colicine M. Tous les mutants de coli V résistant à la colicine C ne produisent cependant pas tous la colicine M. En faisant agir une même dose de colicine C sur différentes subcultures de coli V, nous avons constaté non seulement que le nombre de mutants résistant à la colicine C peut être très différent d'une subculture à l'autre, mais encore que la proportion des mutants produisant la colicine M parmi ces mutants résistants est également très variable. Ceci prouve donc que la mutation amenant la résistance à la colicine C et aussi la mutation déterminant la production de colicine M se produisent spontanément.

Si les mutants produisant la colicine M ont une origine spontanée, comment sont-ils sélectionnés par des agents différents tels que colicine C et phages T.1, T.5 ou T.7? Nous avons constaté que, chez les mutants sélectionnés par les phages T.1, T.5 ou T.7, la résistance à ces phages est toujours associée à la résistance à la colicine C et qu'il n'est dès lors pas étonnant que phages et colicine C sélectionnent les mêmes cellules bactériennes. C'est là un nouvel exemple de résistance croisée spécifique entre colicines et bactériophages (4, 9). La résistance aux phages T.1, T.5 et T.7 est toujours liée à la résistance à la colicine C et à la production de colicine M, tandis que la résistance à la colicine C ne s'accompagne pas nécessairement de résistance aux phages et de la production de colicine M. C'est pourquoi tous les mutants sélectionnés par les phages T.1, T.5 ou T.7 sont également résistant à la colicine C et produisent la colicine M tandis qu'une partie seulement des mutants sélectionnés par la colicine C sont également résistant aux phages T.1, T.5 et T.7 et produisent alors la colicine M.

Puisqu'il existe une résistance croisée spécifique entre colicine C et phages T.1, T.5 ou T.7, et, ainsi que nous l'avons montré précédemment (6, 8), entre phages T.1, T.5 ou T.7 et colicine M, il doit par conséquent y avoir aussi résistance croisée entre colicine C et colicine M. Une résistance croisée entre colicine C et colicine M est difficile à mettre en évidence chez la souche *E.coli φ* dont la sensibilité à la colicine C est faible et spontanément variable mais elle se démontre aisément chez la souche *S.schottmuelleri* V4R nettement sensible à ces deux colicines. En étudiant de nombreuses souches d'Entérobactériacées, nous avons trouvé, à coté de souches insensibles aux colicines C et M et au phage T.1 ou sensibles à un seul de ces trois agents, des souches de quatre types donnant alors les résistances croisées suivantes:

| Sensibilité à | | | Résistance croisée |
|---|---|---|---|
| C | M | T.1 | |
| + | — | + | C et T.1 |
| — | + | + | M et T.1 |
| + | + | — | C et M |
| + | + | + | C, M et T.1 |

La résistance croisée aux colicines C et M et aux phages T.1, T.5 et T.7, montre que ces agents agissent vraisemblablement par l'intermédiaire d'un récepteur commun et nous explique pourquoi la colicine C et les phages T.1, T.5 ou T.7 sélectionnent les mutants de coli V produisant la colicine M. Nous avons en effet constaté par l'étude de plus d'un millier de souches antibiotiques d'Entérobactériacées que ces souches ne sont jamais sensibles à l'antibiotique qu'elles produisent et ne possèdent par conséquent pas le récepteur correspondant. Pour produire la colicine M, les mutants de coli V doivent donc perdre le récepteur correspondant et devenir ainsi résistant non seulement à la colicine M mais aussi à la colicine C et aux phages T.1, T.5 et T.7.

En conclusion, les mutants de *E.coli* V produisant la colicine M apparaissent spontanément puisqu'on les isole sous l'action d'agents aussi différents que colicine C et phages T.1, T.5 ou T.7, et en nombre variable suivant les subcultures considérées. La propriété de produire la colicine M est liée à la résistance aux phages T.1, T.5 et T.7 et à la colicine C, parce qu'elle exige la perte du récepteur à la colicine M, récepteur qui conditionne également la sensibilité aux phages T.1, T.5 et T.7 et à la colicine C.

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# MANIFESTATION OF A LABORATORY EPIZOOTIC OF RABBIT POX BY NON-SPECIFIC STIMULI

by

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One of the difficulties in interpreting the production of experimental disease in laboratory animals, is the exclusion of latent infection in the animal. After the intracerebral inoculation of 75 apparently healthy rabbits with cerebrospinal fluid of 52 patients suffering from multiple sclerosis and other diseases of the central nervous system during one winter of World War II, 46 of the rabbits inoculated presented symptoms of meningoencephalitis (excitement, opisthotonus, grinding of teeths, abundant secretion of saliva, convulsions, paralysis and lethargy). The animals died 1 to 60 days following inoculation. Intracerebral passages were made with the brains of 14 rabbits. All of the 31 rabbits of the second passage died under similar symptoms 2 to 24 days after inoculation. In the third and fourth passage, the time of survival was invariably fixed at 4 days, and 100 % of the animals inoculated died.

Since no bacteria were cultivated from the cerebrospinal fluids of the patients, and from the rabbit brains, the presence of a virus seemed highly probable. As it appeared practically improbable, that this virus would have been present in the cerebrospinal fluid of so many patients suffering from various disorders of the central nervous system, a latent infection of the rabbits was suggested.

In order to test this hypothesis, 12 apparently healthy rabbits from the stock were inoculated intracerebrally with indifferent materials, such as saline, broth, glycerol. Eleven of these rabbits presented the same symptoms as those mentioned above, and died 5 to 21 days following inoculation. Intracerebral passages of these rabbit brains had similair result as the brain-to-brain passages of rabbits inoculated with the cerebrospinal fluids.

When starting these inoculation experiments, the animals used were apparently healthy, but later symptoms of acute respiratory infection with conjunctivitis appeared among the stock rabbits. The epizootic started in one animal house, and spread over 2 others. In some rabbits keratitis, and in the males orchitis was observed, which suggested a disease described by JANSEN (5) as rabbit plague, but which seems to be rabbit pox. Approximately 60 % of the animals died, which is about the same mortality as observed in the rabbits inoculated with cerebrospinal fluid.

On post mortem examination the following lesions were found:

Edematous swelling of the scrotum, which was grown together with the tunica vaginalis communis, and haemorrhagic inflammation of the testicles. Some haemorrhagic-fibrinous exudate in the abdominal cavity. Greyish-white irregularly shaped foci and some subcapsular pox-like lesions in liver and spleen. In the lungs disseminated foci were found. The nasal mucous membrane showed erythematous swelling, and was covered by mucopurulent exudate. On the tongue, one or more typical pox lesions were found. On the skin no lesions were observed.

On microscopic examination, the lesions in the visceral organs appeared to consist of polynuclear and mononuclear cells, sometimes in perivascular arrangement. These foci were similar to those described in the lungs of rabbits with generalized vaccinia by BIJL and FRENKEL (1). The testicles showed a diffuse haemorrhagic inflammatory reaction with extensive necrosis. In the vast majority of the rabbits, infiltrations of the meninges with mononuclear and polynuclear cells were present. The infiltrations were mostly localized near the site of the intracerebral injection. In the same area an inflammatory reaction of the cerebral cortex was found. A more detailed description of the lesion in the central nervous system is given by WENSINCK (13).

The experimental investigation revealed the presence of a virus in the blood, the brains and visceral organs, the nasal secretions and the lesions of the tongue.

Rabbits proved susceptible to corneal, dermal, intradermal, intranasal and intracranial inoculation. After corneal inoculation lesions similar to those produced by vaccinia virus developed. In smears of the corneal surface stained by the methods of HERZBERG and PASCHEN, elementary bodies, and in microscopic slides of the cornea inclusion bodies in the epithelial cells were found. Upon dermal inoculation typical pox lesions with a haemorrhagic and necrotizing reaction, subcutaneous edema, and sometimes generalized vaccinia developed. When introduced intranasally, the virus caused symptoms of acute respiratory disease and conjunctivitis after an average incubation period of 5 days. The intracerebral inoculation produced symptoms of meningoencephalitis and death 4 days following inoculation.

The animal experiments suggested, that the epizootic was due to a neurotropic vaccinia virus, and that it was identical with epizootics of rabbit pox described by others.

Rabbits which had survived the spontaneous disease, and rabbits which had previously been immunized with vaccinia virus proved fully resistant to reinfection with the rabbit pox virus. Since our apparently healthy rabbits at that time might be immune or

TABLE 1.

Cross-immunity experiments by dermal inoculation.

| Animal | First inoculation | | Reinoculation after 15 to 22 days | |
|---|---|---|---|---|
| | Virus | reaction | Virus | reaction |
| Guinea pig 2 | rabbit pox | + | Neurovaccinia | — |
| ,, 3 | ,, | + | ,, | accelerated, allergic |
| ,, 4 | ,, | + | ,, | — |
| ,, 6 | ,, | + | ,, | — |
| ,, 7 | ,, | + | ,, | accelerated, allergic |
| ,, 8 | ,, | + | ,, | accelerated, allergic |
| ,, 9 | ,, | + | ,, | accelerated, allergic |
| ,, 10 | ,, | + | ,, | — |
| ,, 11 | ,, | + | ,, | accelerated, allergic |
| ,, 12 | ,, | + | ,, | accelerated, allergic |
| ,, 23 | ,, | + | ,, | — |
| ,, 32 | ,, | + | ,, | — |
| ,, 33 | ,, | + | ,, | — |
| ,, 35 | ,, | + | ,, | — |
| ,, 37 | ,, | + | ,, | accelerated, allergic |
| ,, 44 | ,, | + | ,, | — |
| ,, 48 | ,, | + | ,, | — |
| ,, 75 | ,, | + | ,, | accelerated, allergic |
| ,, 105 | ,, | — | ,, | + |
| ,, 108 | ,, | + | ,, | — |
| ,, 122 | ,, | + | ,, | — |
| rabbit 1498 | ,, | + | dermal vaccinia | — |
| ,, 1557 | ,, | + | cow pox | — |
| ,, 1341 | dermal vaccinia | + | rabbit pox | accelerated, allergic |
| ,, 1552 | cow pox | + | ,, | — |

latently infected, the cross-immunity experiments were carried out in newly bought rabbits and guinea pigs by dermal inoculation, and from these experiments the close immunological relationship of the rabbit pox virus with vaccinia virus also appeared (table 1).

Like in some of the laboratory epizootics of rabbit pox described by NICOLAU and KOPCIOWSKA (9), GREENE (4), PEARCE, ROSAHN and HU (11), KASAHARA et al. (6), FUST (3) neurovaccinia, with which virus we had been working at that time, might have been the source of infection.

The spread of neurovaccinia virus among rabbits, however, does not constantly result in an epizootic of rabbit pox. LEVADITI and SANCHIS-BAYARRI (8), and DURAN-REYNALS (2) observed in laboratories working with this virus latent infections in rabbits, which only produced immunity, whereas NICOLAU and KOPCIOWSKA (10), and LEVADITI, LÉPINE and SCHOEN (7) observed rabbit pox in contaminated environments after non-specific lowering of resistance, e.g. by depilation and intracerebral inoculation of non-infectious brain suspensions, the latter being similar to our observations. VERLINDE (12) produced a carrier state by subcutaneous inoculation of rabbits with neurovaccinia. When these virus carriers were exposed to non-specific stimuli, i.e. subcutaneous inoculation of fats, glycerol, fresh or autolyzed brain suspensions, most of them died, and neurovaccinia virus could be isolated from the blood, the central nervous system, and the abdominal organs. 14 % of the animals showed rabbit pox and generalized vaccinia.

The animal and cross-immunity experiments seem to leave little doubt, that the virus causing the epizootic of rabbit pox described, is closely related, or perhaps identical with vaccinia virus, and that particularly the pathogenic properties of the virus as determined by experiments in rabbits, reveal the close relationship or identity with neurovaccinia virus.

## Summary.

An epizootic of rabbit pox among laboratory rabbits is described. The animals were probably infected by neurovaccinia used in laboratory experiments. The disease appeared after intracerebral inoculation of cerebrospinal fluid from patients suffering from various disorders of the central nervous system, which indicates, that non-specific stimuli may give rise to a clinical manifestation of

a latent infection, evidence of which was obtained experimentally. The virus isolated possessed the pathogenic and immunologic properties of neurovaccinia virus.

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(Section de Bactériologie et Pathologie expérimentale de l' Institut de Médecine Préventive, Leyden).

# ACTION VIRULICIDE DE QUELQUES SUBSTANCES ANTISEPTIQUES CONTRE LE VIRUS POLIOMYÉLITIQUE MURIN COLUMBIA-SK

par

**A. KLARENBEEK**

(Reçu le 15 mars 1951).

Dans chaque laboratoire, où l'on s'occupe de la recherche des germes pathogènes, il faut se rendre compte de la possibilité d'une infection accidentale, en étudiant le matériel en vitro ou en vivo. Cela peut être une infection directe de l'homme ou une contamination directe par le matériel souillé. C'est pourquoi on trouve presque partout des instructions, mentionnant la manière de stériliser les ustensiles nécessaires pour les recherches et celle de la destruction des animaux infectés, dont on a fait l'examen.

Souvent on peut se servir de l'action destructive des températures très élevées; c'est une méthode simple, de peu de frais et en même temps sûre. Pour la stérilisation des espaces étroits, comme ceux qu'on utilise pour la récolte de virus de l'oeuf fécondé et infecté auparavant, il est quelquefois pratique, de se servir des rayons ultraviolets.

Outre ces méthodes physiques, il sera aussi nécessaire d'appliquer la désinfection chimique. Les substances antiseptiques, qu'on a choisies, doivent être présentes à toute heure et dans une concentration utilisable tout de suite. Le choix de ces substances est différent. Il peut être fixé par le résultat satisfaisant d'expériences préalables, sur leur action stérilisante, qu'on aura faites soi-même, ou dont on aura trouvé les résultats dans les périodiques scientifiques. Il est moins juste de se contenter simplement d'une description des qualités du produit par le fabricant. Il est irrationnel et donc à rejeter de préférer une substance, à cause d'une qualité favorable, comme une odeur agréable, qui n'a aucune importance pour l'action stérilisante.

En général, on ne se rend pas suffisamment compte de l'influence du milieu. Il faut savoir, que la stérilisation est beaucoup plus facile dans un liquide clair et ne contenant le virus dans un état pur et privé de particules organiques du tissu contaminé, que dans une suspension de tissu, riche en virus, mais aussi riche en substance organique. Il y a aussi des produits stérilisants, dont l'action est sérieusement diminuée par la présence de savon. Et enfin il faut se rappeler que les solutions antiseptiques ne gardent pas toutes leur action longtemps. Les produits, par examples, dont l'action stérilisante est due à la contenance en chlore actif, perdent plus ou moins rapidement cette qualité, surtout au contact de l'air.

Dans cet article on trouvera le rapport des résultats des expériences, concernant une comparaison de l'action virulicide de quelques solutions antiseptiques.

Les expériences ont été faites avec le virus poliomyélitique murin, nommé Columbia-SK, qui était extrêmement virulente pour la souris. Des cerveaux de souris, infectées quelques jours auparavant par injection intracérébrale, puis succombées ou sacrifiées dans le stade paralytique, on a préparé une suspension de 5%, par macération minutieuse dans un mortier avec addition d'une solution saline isotonique. Après centrifugation de 10 minutes à 3000 r.p.m. le liquide était séparé du résidu au moyen d'une pipette; cette suspension servit à l' expérience. Elle n'était pas claire ou homogène, mais contenait un grand nombre de particules bien visibles au microscope; elle était virulente, presque toujours en dilutions de $10^{-9}$ jusqu'à $10^{-10}$.

Cette suspension et une solution de la substance antiseptique étaient mélangées de manière à obtenir des concentrations de virus et de la substance antiseptique désirées. Ce mélange était agité rigoureusement pendant 5, 10 ou 20 minutes. Le temps d'agitation écoulé, on dilue une partie du liquide avec la solution saline isotonique. Il faut, que la concentration du virus correspondante sans la substance antiseptique, tue la souris par inoculation intrapéritonéale et qu'en outre, la concentration de la solution antiseptique n'ait pas de qualités toxiques par inoculation intrapéritonéale.

Alors, une fixation préalable de la dose tolérable maxima par voie intrapéritonéale était indispensable. Pour la détermination de la virulence du mélange dilué, on injecte une dose de 0,1–0,2 c.c.

(selon la taille de la souris) chaque fois chez 5 souris. Un groupe de 5 souris pour controler la virulence de la dilution correspondante du virus, doit être ajouté. La mortalité formait la base de l'action virulicide de la substance antiseptique.

Les substances suivantes ont été examinées d'une telle manière: le formol, la chloramine, l'hexachlorophène (G11), le désogen (Geigy), le supérol, le bichlorure de mercure, le furacine et l'hydroxyde de soude.

Les résultats finals ont été résumés dans le tableau suivant. Ils donnent lieu à quelques remarques.

TABLEAU.

Action virulicide contre le virus Columbia-SK.

| Substances antiseptiques (As) | Concentration finale en vitro en % | | Durée de contact en vitro et en minutes | Concentration par inj. intra-pér. en % | | Mortalité en % |
|---|---|---|---|---|---|---|
| | SK | As | | SK | As | |
| Formol | 1 | 1 | 20 | 0,05 | 0,05 | 0 |
| ,, | 1 | 2 | 20 | 0,05 | 0,1 | 0 |
| ,, | 5 | 10 | 5 | 0,05 | 0,1 | 0 |
| Chloramine | 5 | 4 | 5 | 0,05 | 0,04 | 0 |
| Hexachlorophène | 2,5 | 2 | 5 | 0,05 | 0,04 | 0 |
| Désogen (Geigy) | 1 | 5 | 20 | 0,05 | 0,25 | 100 |
| Supérol | 5 | 1 | 20 | 0,05 | 0,01 | 100 |
| Bichlorure de Hg | 5 | 0,1 | 20 | 0,05 | 0,001 | 60 |
| Furacine | 0,1 | 0,047 | 20 | 0,01 | 0,0047 | 100 |
| Hydroxyde de soude | 2,5 | 1 | 20 | 0,05 | 0,02 | 60 |

Une action convenable pouvait être constatée dans les trois premières substances. Une concentration de 10% de formol, préparée d'une solution de formaldehyde, était capable de stériliser en 5 minutes. Avec les solutions de 1—2%, la durée du contact était 20 minutes.

Pour la destruction de virus en suspensions peu homogènes, dans lesquelles la matière infectieuse se trouvera dans un conglomérat de cellules tissulaires, ce qui empéche une attaque vigoureuse, il faut se rendre compte du pouvoir astringent des solutions de formol, par lequel la particule tissulaire et surtout la surface devient plus dure et rigide, à cause de la coagulation de l'albumine cellulaire. Ce fait pouvait être controlé de la manière suivante. Des cerveaux de souris, infectées par le virus Columbia-SK, étaient fragmentés avec un scalpel en petites particules, qui étaient mises dans une solution de formol de 0.1% à —20°C. Après quelques jours le tissu était devenu plus dur et rigide. Néanmoins il était possible d'infecter des souris par injection cérébrale par une suspension diluée, après 22 jours; le virus n'était plus assez virulent après 35 jours.

Les avantages de l'usage du formol sont, sauf l'action virulicide, la stabilité, l'absence d'une couleur désagréable, l'innocuité relative pour la plupart des matériaux et le prix modéré. Les inconvénients sont l'odeur pénétrante, l'irritation des muqueuses de l'oeil et du nez et l'action plus ou moins caustique sur la peau, au contact direct et intensif.

Les solutions de chloramine de 4%, préparées avec la poudre, contenant 20,3% de chlore actif, peuvent détruire le virus en 5 minutes. La concentration est beaucoup plus élevée que celle, qu'on utilise pour la stérilisation bactérielle de l'eau (1—3). La différence est causée par la présence d'un grand nombre de particules tissulaires dans les suspensions de cerveaux.

Les chloramines ont donc montré une action appréciable et ont en outre comme avantage: l'innocuité pour la peau ou pour la muqueuse, l'absence de couleur, une toxicité très faible et le prix modéré. La réduction progressive du taux de chlore actif dans les solutions, surtout en communication directe avec l'air, est un inconvénient. Cependant, l'activité des chloramines est beaucoup plus durable, que celle des hypochlorites, qui sont aussi des substances bien connues.

Les expériences avec le hexachlorophène (G11) ont montré, que la concentration de 2% donnait déja une stérilisation complète en 5 minutes. Le G11 est un phénol chloriné, pourvu d'une action bactéricide remarquable. On en a propagé l'application surtout pour la désinfection de la peau (chirurgie, dermatologie), parce que l'action bactéricide ne diminue pas en présence de savon. Le G11 a d'autres qualités agréables: il ne donne pas d'irritation de la peau ou des muqueuses, la toxicité est presque négligeable, la solution n'a ni odeur, ni couleur gènante. Il sera intéressant, si les expériences avec des autres virus pourront montrer la même activité rigoureuse comme chez le virus Columbia-SK. La discussion des autres substances examinées peut être courte.

Le désogen (Geigy), employé a 0,5% de solution aqueuse contre les infections bactérielles, ne montrait aucun pouvoir virulicide, pas même avec les solutions de 5% et le contact de 20 minutes.

Le superol, un dérivat de l'oxychinoline, échouait de même en concentration de 1% et contact pendant 20 minutes.

La furacine, le représentant bien connu du groupe de nitrofurane, recommendée vivement comme substance antiseptique (unguent 0,2%), est restée complèment en défaut en solution aqueuse presque saturée (0,042%) et contact de 20 minutes.

L'hydroxyde de soude, qui rend des services appréciables à la campagne par la destruction de virus de la fièvre aphtheuse et en solutions de 0,5–1,0%, ne donna aucun résultat contre le virus Columbia-SK en solution de 1% et après 20 minutes de contact.

## Conclusion.

En cas d'infection accidentale de personnes ou du matériel dans le laboratoire avec des virus, il sera indispensable de choisir la substance virulicide avec soin. La concentration doit être beaucoup plus élevée que celle, qui c'est montrée mortelle pour les virus, plus ou moins purifiés. C'est surtout la contenance tissulaire, qui protège le virus et retarde sa destruction. La pénétration de la substance antiseptique, comme le formol et le bichlorure de mercure est en outre ralentie par la coagulation de l'albumine cellulaire. Le formol, la chloramine et l'hexachlorophène se sont montrés utilisable. C'est surtout l'action virulicide rigoureuse de l'hexachlorophène, qui donne des perspectives intéressantes.

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# EXAMEN BACTÉRIOLOGIQUE DE QUELQUES DÉRIVÉS DE L'ACIDE PTÉROYLGLUTAMIQUE

par

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(Reçu le 18 mai 1951).

Afin de synthétiser des antagonistes nouveaux de l'acide ptéroylglutamique, l'un de nous (A.C.H.) a préparé des dérivés de cet acide en introduisant des substituants dans le noyau benzénique. Les détails des recherches chimiques, exécutées dans le laboratoire de chimie organique de l'Université de Groningue, seront publiés dans le Recueil des Travaux chimiques des Pays Bas [1]).

Nous décrivons ci-dessous l'examen de leur influence sur la croissance de *Streptococcus faecalis* R. (*Streptococcus lactis* R.; A.T.C.C. 8043) [2]) dans un milieu synthétique.

La culture de cette microbe est conservée à 4° C. dans ,,Bacto Micro Assay Culture Agar'' [3]). On la repique chaque mois. L'ensemencement se fait en profondeur.

Le jour précédant la préparation d'une expérience la bactérie est inoculée dans 10 $cm^3$ de ,,Bacto Micro Inoculum Broth'' [3]) et cultivée à 37° C. Après 24 heures la culture est centrifugée et le liquide est décanté. Le sédiment du *Streptococcus lactis* R. est émulsionné dans de l'eau salée physiologique stérile. La suspension microbienne est diluée jusqu'à un état trouble à peine visible; alors elle contient environ $10^8$ microbes par $cm^3$. Chaque tube de culture est ensemencée avec une goutte de cette suspension.

Pour milieu d'essai nous avons employé une solution de ,,Dehydrated Folic Acid Assay Medium'' [3]). Dans une série de tubes de culture on introduit 5 $cm^3$ de ce liquide (concentration double), la solution de l'acide ptéroylglutamique ou d'un des ses dérivés (en différentes concentrations) et de l'eau distillée stérile (volume total 10 cm$^3$); après avoir agité les tubes, on ajoute la bactérie. Les tubes sont placés dans une étuve à 37° C. et le lendemain leur état trouble est examiné à l'œuil nu.

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[1]) H. J. Backer et A. C. Houtman, publication inédite.

[2]) Le professeur B. C. P. Jansen (Amsterdam) a eu l'obligeance de nous en fournir une culture pure.

[3]) Produit des laboratoires "Difco" à Detroit (Michigan, U.S.A.).

Afin de pouvoir juger l'action des substances ajoutées sur la croissance des bactéries, nous nous sommes servis d'un système simple d'évaluation.

Lorsque la liqueur de culture reste claire, l'absence de croissance est indiquée par le signe —. Une croissance faible est désignée par ±. Les états troubles plus prononcés sont indiqués successivement par +, 2+, 3+, 4+ et 5+.

De temps en temps on contrôle, au moyen de préparations colorées selon la méthode de Gram, si l'état trouble est causé par des streptocoques Gram-positifs et non pas par d'autres microbes.

Les composés suivants ont été examinés:

Acide ptéroylglutamique, préparé dans le laboratoire (A.P.G.).
,, folique de l'industrie pharmaceutique (A.F.).
,, 2'-fluoro-ptéroylglutamique (2'-F).
,, 2'-chloro- ,, (2'-Cl).
,, 2'-méthyl- ,, (2'-$CH_3$).
,, 3'-méthyl- ,, (3'-$CH_3$).
,, 2'-méthoxy- ,, (2'-$OCH_3$).
,, 3'-méthoxy- ,, (3'-$OCH_3$).

(Le symbole 2' indique que le substituant se trouve en position 2 du noyau benzénique si le groupe CO occupe la position 1).

## A. ACTIVITÉ COMME FACTEUR DE CROISSANCE.

L'acide ptéroylglutamique (A.P.G.) préparé dans le laboratoire se comporte comme l'acide folique (A.F.) de l'industrie pharmaceutique. L'acide 2'-fluoro-ptéroylglutamique (2'-F) présente la même action. (L'identité de ce dernier acide n'est pas établie avec certitude absolue).

| γ/10 cm$^3$ | 0.000 | 0.001 | 0.002 | 0.004 | 0.006 | 0.008 | 0.010 |
|---|---|---|---|---|---|---|---|
| A. P. G. | — | + | 2+ | 3+ | 4+ | 5+ | 5+ |
| A. F. | — | + | 2+ | 3+ | 4+ | 5+ | 5+ |
| 2'-F | — | + | 2+ | 3+ | 4+ | 5+ | 6+ |

Une quantité de 0.001 γ d'acide par tube correspond à une concentration de 1 sur $10^{10}$.

Parmi les cinq autres acides ptéroylglutamiques, seuls les dérivés 2'-chloro et 3'-méthoxy peuvent remplacer un peu l'acide A.P.G. comme facteur de croissance en concentration assez élevée. Mais la croissance des bactéries sous l'influence de ces substances est beaucoup moins abondante qu'avec A.P.G. Les trois autres composés n'ont pas cette action.

| $\gamma/10$ cm$^3$ | 0.002 | 0.050 | 0.100 | 1.00 | 5.0 | 10.0 | 50.0 |
|---|---|---|---|---|---|---|---|
| 2'-$CH_3$ | | — | | — | | — | |
| 3'-$CH_3$ | | — | | — | | — | |
| 2'-$OCH_3$ | — | — | — | — | — | — | — |
| 3'-$OCH_3$ | | — | | — | | + | |
| 2'-Cl | — | — | — | — | ± | 2+ | 2+ |

## B. Action bactériostatique.

Afin d'examiner l'activité antagoniste vis à vis de l'acide ptéroylglutamique, on introduit dans tous les tubes 0.002 $\gamma$ de cet acide avant d'ajouter les dérivés à éprouver. Cette quantité suffit à causer une croissance appréciable. Les composés qui freinent la croissance ont été examinés ensuite en présence d'une concentration plus élevée d'acide ptéroylglutamique.

Acide 2'-méthyl-ptéroylglutamique.

| A.P.G. $\gamma/10$ cm$^3$<br>2'-$CH_3$ $\gamma/10$ cm$^3$ | 0.002 | 0.002<br>0.10 | 0.002<br>0.50 | 0.002<br>1.00 | 0.002<br>2.00 | 0.002<br>5.00 | 0.002<br>10.00 |
|---|---|---|---|---|---|---|---|
| après 15 h | 2+ | 2+ | + | ± | ± | ± | — |
| après 24 h | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ | — |

| A.P.G. $\gamma/10$ cm$^3$<br>2'-$CH_3$ $\gamma/10$ cm$^3$ | 0.020<br>1.0 | 0.020<br>4.5 | 0.020<br>10.0 | 0.020<br>20.0 | 0.020<br>45.0 |
|---|---|---|---|---|---|
| après 15 h | 3+ | 3+ | 3+ | 4+ | 4+ |
| après 24 h | 4+ | 4+ | 4+ | 4+ | 4+ |

Ce composé (2'-$CH_3$) est faiblement bactériostatique. Pour des concentrations faibles de A.P.G. l'indice antibactérien est d'environ 5000. Nous entendons par indice antibactérien le quotient des concentrations d'antagoniste et d'acide ptéroylglutamique, pour lesquelles la croissance est juste empêchée. Si ce quotient est constant pour de différentes concentrations de l'antagoniste on parle d'un antagonisme compétiteur.

Acide 3′-méthyl-ptéroylglutamique.

| A.P.G. $\gamma/10$ cm$^3$<br>3′-$CH_3$ $\gamma/10$ cm$^3$ | 0.002 | 0.002<br>0.005 | 0.002<br>0.010 | 0.002<br>0.050 | 0.002<br>0.100 | 0.002<br>0.50 | 0.002<br>2.00 |
|---|---|---|---|---|---|---|---|
| après 15 h | 2+ | 2+ | 2+ | ± | ± | — | — |
| après 24 h | 2+ | 2+ | 2+ | ± | ± | — | — |

| A.P.G. $\gamma/10$ cm$^3$<br>3′-$CH_3$ $\gamma/10$ cm$^3$ | 0.020 | 0.020<br>0.050 | 0.020<br>0.100 | 0.020<br>0.50 | 0.020<br>1.00 | 0.020<br>5.0 | 0.020<br>20.0 |
|---|---|---|---|---|---|---|---|
| après 15 h | 4+ | 3+ | 4+ | 4+ | ± | — | — |

| A.P.G. $\gamma/10$ cm$^3$<br>3′-$CH_3$ $\gamma/10$ cm$^3$ | 0.20 | 0.20<br>0.50 | 0.20<br>1.00 | 0.20<br>5.00 | 0.20<br>10.0 | 0.20<br>50.0 |
|---|---|---|---|---|---|---|
| après 15 h | 4+ | 3+ | 3+ | 3+ | + | — |

Pour toutes les concentrations de A.P.G. l'indice antibactérien est d'environ 100. Ce composé bactériostatique puissant (3′-$CH_3$) présente clairement le caractère d'antagoniste compétiteur.

Acide 2′-méthoxy-ptéroylglutamique.

| A.P.G. $\gamma/10$ cm$^3$<br>2′-$OCH_3$ $\gamma/10$ cm$^3$ | 0.002 | 0.002<br>0.020 | 0.002<br>0.100 | 0.002<br>0.200 | 0.002<br>0.40 | 0.002<br>0.50 | 0.002<br>1.00 | 0.002<br>2.00 |
|---|---|---|---|---|---|---|---|---|
| après 15 h | 2+ | 2+ | 2+ | + | ± | ± | — | — |
| après 24 h | 3+ | 3+ | 2+ | 2+ | 2+ | + | ± | — |

| A.P.G. $\gamma/10$ cm$^3$<br>2′-$OCH_3$ $\gamma/10$ cm$^3$ | 0.006 | 0.006<br>0.020 | 0.006<br>0.250 | 0.006<br>0.30 | 0.006<br>0.60 | 0.006<br>1.20 | 0.006<br>3.0 | 0.006<br>6.0 |
|---|---|---|---|---|---|---|---|---|
| après 15 h | 3+ | 3+ | 2+ | 2+ | 2+ | + | — | — |
| après 24 h | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | ± | — |

Ce composé (2′-$OCH_3$) agit également comme antagoniste compétiteur. L'indice antibactérien est d'environ 500.

Acide 3′-méthoxy-ptéroylglutamique.

| A.P.G. $\gamma/10$ cm$^3$<br>3′-$OCH_3$ $\gamma/10$ cm$^3$ | 0.002 | 0.002<br>0.010 | 0.002<br>0.100 | 0.002<br>0.50 | 0.002<br>2.0 | 0.002<br>10.0 |
|---|---|---|---|---|---|---|
| après 15 h | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ |
| après 24 h | 2+ | 2+ | 2+ | 2+ | 2+ | 2+ |

Ce dérivé n'a aucune action antagoniste vis à vis de A.P.G.

Acide 2′-chloro-ptéroylglutamique.

| A.P.G. $\gamma/10\ cm^3$<br>2′-Cl $\gamma/10\ cm^3$ | 0.002 | 0.002<br>0.020 | 0.002<br>0.080 | 0.002<br>0.50 | 0.002<br>2.0 | 0.002<br>10.0 | 0.002<br>50.0 |
|---|---|---|---|---|---|---|---|
| après 15 h | 2+ | 2+ | + | ± | ± | ± | ± |
| après 24 h | 3+ | 2+ | 2+ | 2+ | + | 2+ | 2+ |

L'action antagoniste est négligeable. Les concentrations élevées causent un état trouble moins homogène. L'examen microscopique démontre une certaine dégénération des bactéries.

Puisque l'acide *p*-aminobenzoïque, facteur de croissance pour certaines bactéries, freine la croissance en concentration élevée, il nous a paru intéressant d'examiner si A.P.G. pourrait présenter la même activité.

| A.P.G. $\gamma/10\ cm^3$ | 0.001 | 0.002 | 0.004 | 0.006 | 0.008 | 0.010 | 10.0 | 50.0 |
|---|---|---|---|---|---|---|---|---|
| après 15 h | + | 2+ | 3+ | 4+ | 5+ | 5+ | 5+ | 5+ |

Même une concentration de $50\ \gamma/10\ cm^3$, soit 6000 fois la quantité nécessaire pour la croissance maximale, n'a aucune influence bactériostatique.

## Résumé.

Les dérivés 3′-méthyl, 2′-méthoxy et 2′-méthyl de l'acide ptéroylglutamique sont des antagonistes compétiteurs de cet acide. Il exercent une action bactériostatique sur *Streptococcus lactis* R. (A.T.C.C. 8043). Leurs indices antibactériens sont 100, 500 et 5000 respectivement.

Le (probable) dérivé 2′-fluoro peut remplacer l'acide ptéroylglutamique comme facteur de croissance. Le dérivé 3′-méthoxy n'a qu'une action faible à cet égard et l'action du dérivé 2′-chloro est douteuse.

(From the Bacteriological-Serological Laboratory of the State University of Groningen).

# QUANTITATIVE COMPLEMENT FIXATION

## I. STANDARDIZATION OF COMPLEMENT, SHEEP'S BLOOD AND AMBOCEPTOR

by

**H. H. COHEN**

(Received April 28, 1951).

In an investigation on the diagnostic and prognostic significance of the complement fixation in tuberculosis, the necessity of standardization of the reagents, used in this reaction, was felt. For this purpose it is essential to use accurate titration methods. In the complement reaction most laboratories use the amount of complement, which gives rise to 100% lysis of a given haemolytic system.

The point at which this occurs, is difficult to ascertain with accuracy. From the haemolysis curve it is, however, seen that the point at which 50% of the erythrocytes are haemolysed is easier to determine. The most accurate investigations by means of the amount of complement which haemolyses 50% of the cells ($x$ — 50 EH), were carried out by Wadsworth, Maltaner and Maltaner (10, 6, 11, 12). It was found that the formula of von Krogh was approximately applicable to the curve of haemolysis. If $x$ is the amount of complement, which gives rise to a percentage haemolysis $y$, then the following relationship exists between $x$ and $y$:

$$\log x = \log K + \frac{1}{n} \log \frac{y}{1-y}$$ ($n$ = constant and $K$ = constant. $K$ equals the amount of complement, giving rise to 50% haemolysis).

It is clear, that this formula will describe a straight line. In practise it is found, that the estimation of the 50 EH complement by means of the hand-drawn line through the given points, gives inaccurate results. In an attempt to overcome this difficulty, Kent *et al.* (4) constructed a graph from which, by means of the percentage haemolysis, found in the different tubes ($y$), the amount of complement, giving 50% haemolysis can be read off directly. With the aid of lyophil dried complement, amboceptor and sheep's blood, which was collected in modified ALSEVERS solution [1]), they estimated the 50% unit and the value $\frac{1}{n}$ from the formula of KROGH, every day for a number of weeks.

They accomplished this by applying the method of least squares to the formula of VON KROGH in which they substituted the values, found by them for $x$ and $y$.

But if: $\log x = \log K + \frac{1}{n} \log \frac{y}{1-y}$ and if we let:

$$\log x = X \text{ and: } \log \frac{y}{1-y} = Y,\ \log K = a \text{ and: } \frac{1}{n} = b$$

then:

$$X = Na + bY$$

$$XY = aY + bY^2.$$

($N$ equals the number of tubes, in which the partial haemolysis is measured).

The unknown $a$ and $b$ can be calculated from these formulae. They found a value for $x$ (50 EH) between 0.354 ml — 1/100 and 0.418 ml — 1/100. The value of $\frac{1}{n}$ was fairly constant (0.141 — 0.182, average 0.152). By means of these values they calculated the volume factors by calculating the value of $K$ in the formula of VON KROGH for different values of $y$ when they let $x = 1$.

From their findings it can be seen that sheep's erythrocytes, stored in ALSEVERS solution, remain constant for a period of 26 days. It seemed important to us to investigate, whether this method could be easily applied in practice in this country, and whether it would be possible to carry out the reaction so, that $x$ (50 EH) remains constant for a number of weeks. We would then be able to carry out a quantitative complement fixation reaction for a number of weeks, without being compelled to let it be preceded by a complement titration every day.

The major difficulties are particularly technical. These concern:

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[1]) 100 ml of ALSEVERS solution contains 2.05 g glucose, 0.8 g sodium citrate, 0.42 g sodium chloride and 0.055 g citric acid, pH = 6.1. Sterilized at 110° C. for 30 minutes. The solution should not caramellize.

1) The lyophil drying. Lyophil dried complement is commercially unobtainable in this country. Although the Bacteriological-Serological Laboratory at Groningen has a small apparatus for lyophil drying at its disposal, this method is very time consuming: smaller laboratories are not helped by this. In the literature (RICHARDSON (9), ORPWOOD PRICE (8)) there are favourable reports on a simple chemical method of conserving complement. We decided to investigate the value of this complement, using the method of KENT.

2) Colorimeter. The Coleman Junior Spectrometer, used by KENT *et al.* is very expensive. I decided, that the least expensive photo-electric colorimeter, known to us i.e. the Eel colorimeter [1]), was suitable for my purpose. This colorimeter when fully equipped, costs approximately *ƒ* 500.

## MATERIAL AND METHODS.

Unless expressly stated, I have used the material and methods, used by KENT *et al.* (4).

*a*) Dilution fluid, consisted of physiological saline solution buffered with 0.005 M phosphate.

*b*) Sheep's blood. This was collected sterile with an equal volume of ALSEVERS solution in a container. The contents of the container was divided between small tubes (under sterile circumstances) and stored at + 4° C.

Before the reaction, the volume of the centrifuged cells was determined. This was accomplished by marking the volume on the tube, removing the cells from the tube, letting the tube empty for a few minutes on filter paper and then filling it up to the mark with water from a burette. The cells were diluted 50 times with buffered saline solution.

*c*) Amboceptor. Through the kindness of Dr J. H. BEKKER, head of the Bacteriological Department of the State Institute of Public Health at Utrecht, I obtained new directions of the New York State Department of Health. Very good amboceptors can be prepared by immunizing rabbits with sheep's serum and sheep's erythrocytes. These amboceptors can be stored for a long period of time in an equal volume of glycerol at 4° C. The amboceptor is used at its optimal dilution, i.e. that dilution at which any increase of amboceptor does not decrease the $x$ (50 EH) dose of the complement (DALLDORF (2), KENT (5)). In practice it is found, as reflected by the results in table 1), that this is not absolutely true. Yet fairly accurate results could be obtained by this method.

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[1]) Evans Electoselenium Ltd, Harlow, Essex, England.

In the first series of results obtained by us, as shown in table 1, the amboceptor was each time diluted to the required degree. An equal volume of erythrocyte suspension was added to this while being shaken. The sensitized blood was left in the incubator at 37° C. for 10 minutes. Later it was found, following the example of KENT *et al.* (4), that is was equally possible to store the amboceptor in a dilution of 1/100 for one week at 4° C. By means of weighed pipettes, 20 ml amboceptor solution and 20 ml of 2% erythrocyte suspension were separately introduced into two tubes. The contents of the two tubes were mixed by rapidly pouring them ten times from one tube into the other, commencing with the diluted amboceptor. After standing for 10 minutes, the sensitized blood is ready for use.

Table 1.

| complement (1/106) [1] (in ml) | amboceptor dilution (0.4 ml) | 2% sheep's blood (in ml) | buffered saline solution (in ml) | % haemolysis after 30 minutes at 37° C. |
|---|---|---|---|---|
| 0.4 | 1/250 | 0.4 | 0.8 | 45 % |
| ,, | 1/333 | ,, | ,, | 44.5% |
| ,, | 1/500 | ,, | ,, | 42 % |
| ,, | 1/666 | ,, | ,, | 38 % |
| ,, | 1/1000 | ,, | ,, | 34 % |
| ,, | 1/1333 | ,, | ,, | 34 % |
| ,, | 1/2000 | ,, | ,, | 29.5% |
| ,, | 1/4000 | ,, | ,, | 18 % |

[1]) This dilution is ascertained by first performing a complement titration with an amboceptor dilution of 1/250 and then calculating the $x$ (50EH) complement from this.

It is seen from table 1 that strictly speaking, no point is reached at which the erythrocytes were maximally saturated by means of this very good amboceptor. In this instance we used an amboceptor dilution of 1/500, as its results were approximately the same as those of an amboceptor dilution of 1/250.

*d*) Complement. This was stored as described by RICHARD-

SON (9). For this purpose three solutions were prepared in 5.4 M saturated sodium chloride solution (36 times isotonic) *viz.*,

Solution A: M/4 boric acid (1.55 g in 100 ml).

Solution B: M/2 sorbite plus M/8 sodium azide (9.55 g sorbite and 0.81 g sodium azide in 100 ml).

Solution C: M/8 sodium azide (0.81 g in 100 ml).

To 8 volumes of fresh guinea-pig serum is added one volume solution A, 1/2 volume solution B, and 1/2 volume solution C. The complement, conserved in this way, is stored in the dark. When using it, one volume of conserved complement is diluted with 7 volumes of distilled water, whereby a complement dilution of 1/10, which is isotonic, will be obtained.

*e*) Glass apparatus. Burettes were used when adding amounts in excess of one ml. Dilution was carried out by means of weighed pipettes. The glass apparatus was cleaned with a solution of potassium bichromate in sulphuric acid. Great care was taken in rinsing the apparatus.

## OWN INVESTIGATIONS.

### Photo-electric colorimeter.

For this investigation it had to meet the following requirements:

1) It had to be possible to measure amounts of approximately one ml. The Eel colorimeter was delivered with tubes of 7,4 and 1 ml.

2) The colorimeter had to give constant results from day to day. For this purpose, a potassium bichromate solution was titrated daily (gelatine filter 625, allows light of 540 m$\mu$ wave length to pass).

As indicated in Table 2, the average for ten days is 47.1. Standard error of measurements 0.4. Therefore the Eel colorimeter gives sufficiently constant values for the purpose of this investigation.

3) In the area measured, there must be an absolute ratio between the amount of freed haemoglobin and the extinction (log of the percentage of light, allowed to pass). As the Eel colorimeter has a logarithmic scale, this percentage can be read off directly. By

Table 2.

| Date | Galvanometer values |
|---|---|
| 19/3 | 47.1 |
| 20/3 | 47.2 |
| 21/3 | 46.8 |
| 22/3 | 47.0 |
| 23/3 | 47.7 |
| 24/3 | 47.0 |
| 27/3 | 46.5 |
| 28/3 | 47.2 |
| 29/3 | 47.0 |
| 30/3 | 47.3 |

first setting the galvanometer at zero by means of a tube with distilled water and then measuring the galvanometer deflections for a tube with 100% haemolysis, one can, if the colorimeter meets the above requirements, calculate directly $y$ = the percentage haemolysis, by means of the values, found in the tubes with partial haemolysis. In order to check the galvanometer deflections with different amounts of haemoglobin, I devised the following experiment.

Table 3.

|  | Complement 1/21 (in ml) | 2% sheep's erythrocytes sensitized with amboceptor 1/500 (in ml) | saline solution (in ml) | % haemolysis | Galvanometer readings |
|---|---|---|---|---|---|
|  | — | 0.8 | 1.2 | 0 | 0 |
| 1 | 0.3 | 0.2 | 1.5 | 100 | 11.6 |
| 2 | ,, | 0.4 | 1.3 | 100 | 22.0 |
| 3 | ,, | 0.6 | 1.1 | 100 | 33.5 |
| 4 | ,, | 0.8 | 0.9 | 100 | 44.7 |

One would, accepting the value in tube 4, as 44.7, expect the reading 11.2 in tube 1, the value 22.4 in tube 2 and the value 33.5 in tube 3. The results obtained are within the limits of the expected error. Therefore the extinction values which are obtained, can be converted directly into percentage haemolysis by means of the value for 100% haemolysis.

Therefore the Eel photo-electric colorimeter meets the requirements made upon it for the purpose of this investigation. It is also possible to standardize the 2% sheep's erythrccyte suspension colorometericaly. For this purpose 1 ml of this suspension haemolyzed with 14 ml of a 0.1% solution of sodium carbonate in a 7 ml tube is measured at 540 m$\mu$ against distilled water as control. 35 ± 1.0 is accepted as the reading of the galvanometer deflection. Possible corrections are made by adding saline solution or removing it from the suspension by centrifuging, in amounts determined by the formula $V_2 = \frac{V_1 \times OD_1}{35.0}$.

In this formula $V_1$ equals the volume of the suspension giving in the colorimeter the first determination $OD_1$, $V_2$ is the volume of the suspension after correction. Finally 35.0 ± 1.0 is accepted.

## TITRATION TECHNIQUE AND RESULTS.

The titration technique was exactly the same as that, used by KENT *et al.* (4). Every morning a standard solution of the most suitable complement dilution was prepared, in this instance, here published, it was 1/27. A portion of this was diluted 1/5 with buffered saline solution. The reagents were added to each other in KAHN tubes in the following order: *a.* complement dilution, *b.* buffered saline solution, *c.* sensitized erythrocytes.

The tubes were shaken and corked. After 15 minutes in a water bath at 37° C. they were shaken once again and after a total of 30 minutes they were rapidly centrifuged. After this the % haemolysis (*y*) was calculated by means of the colorimeter, as described above (filter 625, 540 m$\mu$). A tube without complement, in which the cells naturally did not haemolyze, was used as control. A complement dilution of 1/27 was used in three tubes. Complete haemolysis occurred in these. The average of the three galvanometer readings found for these tubes, was accepted as the 100% standard for the calculation of the partial haemolysis values, *y*.

In order to check the influence of pipetting and measuring errors, the following plan was adopted. On one day one haemolytic system and one complement dilution were prepared. Four titrations were carried out with these. From the *y*-values, obtained from each titration, the value of *K* and 1/*n* were calculated by the method of least squares. Only the *y*-values between 20% and 80% were used for this purpose, as the formula of von Krogh is said to be too inaccurate for values outside these limits (Kent *et al.* (4), and Waksman (13)). From all data the daily average for both values, in addition the standard error of $K$, was determined.

On the following day four haemolytic systems and four complement-dilutions were made in exactly the same way and as accurately as possible. Four titrations were carried out with these. In this way four values for $K$ and $1/n$ were obtained in addition to the daily average for both values and the standard error of $K$.

All in all, four titrations were carried out daily for 12 consecutive

Table 4.

Four titrations with four different haemolytic systems on one day.

| date | no. | value of (50EH) in ml 1/135 | value of $\frac{1}{n}$ | average value of $\frac{1}{n}$ [1]) | average $x$ (50EH) from 4 readings [1]) | standard error |
|---|---|---|---|---|---|---|
| 13/2 | 1 | 0.402 | 0.190 | 0.186 | 0.408 | 0.0074 |
| | 2 | 0.408 | 0.185 | | | |
| | 3 | 0.405 | 0.179 | | | |
| | 4 | 0.419 | 0.205 | | | |
| 15/2 | 1 | 0.488 | 0.186 | 0.180 | 0.467 | 0.0207 |
| | 2 | 0.483 | 0.164 | | | |
| | 3 | 0.447 | 0.170 | | | |
| | 4 | 0.451 | 0.227 | | | |
| 17/2 | 1 | 0.415 | 0.164 | 0.184 | 0.422 | 0.0113 |
| | 2 | 0.422 | 0.173 | | | |
| | 3 | 0.413 | 0.174 | | | |
| | 4 | 0.438 | 0.184 | | | |
| 20/2 | 1 | 0.394 | 0.190 | 0.172 | 0.400 | 0.0097 |
| | 2 | 0.414 | 0.170 | | | |
| | 3 | 0.401 | 0.160 | | | |
| | 4 | 0.393 | 0.164 | | | |
| 22/2 | 1 | 0.410 | 0.155 | 0.159 | 0.407 | 0.0120 |
| | 2 | 0.423 | 0.164 | | | |
| | 3 | 0.399 | 0.167 | | | |
| | 4 | 0.397 | 0.167 | | | |
| 24/2 | 1 | 0.413 | 0.154 | 0.182 | 0.406 | 0.0126 |
| | 2 | 0.420 | 0.205 | | | |
| | 3 | 0.392 | 0.195 | | | |
| | 4 | 0.400 | 0.183 | average standard error: | | 0.0123 |

[1]) Determined by applying the method of least squares to the data, obtained from 4 titrations on the same day.

days. An absolute average with its standard error were calculated from the 12 daily averages. If the obtained differences between the daily averages of $K$ were due to measuring and pipetting errors, then the standard error of the absolute average should be $\sqrt{4} \times$ as small as the average value of the 6 standard errors, obtained on those days, when I titrated with 4 separate systems 1).

Table 5.

Four titrations with one haemolytic system on one day.

| date | no. | value of (50EH) in ml 1/135 | value of $\frac{1}{n}$ | average value of $\frac{1}{n}$ *) | average $x$ (50EH) from 4 readings | standard error |
|---|---|---|---|---|---|---|
| 14/2 | 1 | 0.469 | 0.155 | 0.165 | 0.475 | 0.0065 |
| | 2 | 0.483 | 0.185 | | | |
| | 3 | 0.471 | 0.170 | | | |
| | 4 | 0.478 | 0.166 | | | |
| 16/2 | 1 | 0.385 | 0.170 | 0.179 | 0.382 | 0.0029 |
| | 2 | 0.382 | 0.186 | | | |
| | 3 | 0.378 | 0.181 | | | |
| | 4 | 0.382 | 0.171 | | | |
| 18/2 | 1 | 0.411 | 0.172 | 0.169 | 0.408 | 0.0045 |
| | 2 | 0.412 | 0.168 | | | |
| | 3 | 0.402 | 0.169 | | | |
| | 4 | 0.407 | 0.169 | | | |
| 19/2 | 1 | 0.448 | 0.173 | 0.175 | 0.442 | 0.0056 |
| | 2 | 0.444 | 0.170 | | | |
| | 3 | 0.440 | 0.181 | | | |
| | 4 | 0.435 | 0.182 | | | |
| 21/2 | 1 | 0.404 | 0.234 | 0.191 | 0.408 | 0.0056 |
| | 2 | 0.413 | 0.168 | | | |
| | 3 | 0.402 | 0.178 | | | |
| | 4 | 0.404 | 0.185 | | | |
| 23/2 | 1 | 0.336 | 0.176 | 0.159 | 0.339 | 0.0028 |
| | 2 | 0.342 | 0.163 | | | |
| | 3 | 0.337 | 0.155 | | | |
| | 4 | 0.340 | 0.167 | average standard error: | | 0.0046 |

*) Determined by applying the method of least squares to the data, obtained from 4 titrations on the same day.

Average $x$ (50EH) value for the 12 groups: 0.413 with a standard error = = 0.0357. Average $1/n$ value for the 12 groups = 0.175.

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1) The standard error of an average is $\sqrt{n}$ times as small as the standard error of the values measured and every daily average was obtained from four titration values.

With the aid of this variation analysis (Bok (1)), we attempted to analyze the values obtained (tables 4 and 5).

## Discussion of results.

From the findings it can be seen that the fairly marked discrepancies towards both extremes in the two series on 14/2, 15/2 and 23/2 can certainly not be explained by pipetting errors. The variation analysis corroborates this.

The standard error of the average should not exceed $\frac{0.0123}{\sqrt{4}} = 0.0061$ in order to explain the differences detected. It is 0.0357 i.e. 5.8 times as great.

From the table, which, with a number of estimations ($N_1$ and $N_2$), shows the likelihood that the observed ratio between two standard errors is due to chance, it follows that at $N_1 = 4 - 1 = 3$ and $N_2 = 3 \times 12 = 36$, the possibility that the observed variation is due to measuring and pipetting errors is less than 1/1000.

If the results are more closely examined it is, however, seen that these differences which are mainly due to 3 of the 12 in the series, occur arbitrarily. From these measurements it can therefore be concluded that the complement, amboceptor and sheep's erythrocytes retain a constant value for at least a fortnight under the given circumstances.

It is however, not possible with these reagents to keep the $x$ (50 EH) dose constant from day to day.

From the investigations of Eagle and Brewer (3) it was found that the speed of lysis of a cell-amboceptor-complement system depends more on the amount of amboceptor fixed to the cells, than on the total amount of amboceptor, present. If $A$ equals the total amount of added amboceptor, then the amount of amboceptor $a$ utilized by the cells is determined by the following formula: $a = \eta A$. $\eta$ is a constant and varies from 0.25 to 0.75.

The observed differences can probably be ascribed to a day to day variation of $\eta$. In this instance a difference in the speed at which the erythrocytes are added to the amboceptor could play a part because a different pipette was used for this purpose every day.

A second possibility is, that traces of calcium and magnesium ions, or daily differences in salt concentrations, could make the complement more or less active from day to day. From the investigations of MAYER *et al.* (7) it has actually become apparent, that the action of complement is enhanced by a slight concentration of magnesium and calcium ions. It is self evident, that these factors will form the subject of another investigation.

The average daily value of $1/n$ in the formula of VON KROGH varied between 0.159 and 0.191 (KENT: 0.141 — 0.182) with an average of 0.175 (KENT: 0.152).

## Summary.

In agreement with the investigations of KENT *et al.* (4), it was found that blood collected in a modified ALSEVERS solution remains constant for a period of at least 14 days.

We also succeeded in proving the same with regard to complement, stored according to RICHARDSON (9) and with regard to amboceptor prepared according to the directions of the New York State Department of Health and stored in an equal volume of glycerol at 4° C.

The cheap Eel colorimeter proved very suitable for the standardization of the erythrocyte-suspension and the measurement of the percentage of haemolysis.

Success was not obtained by means of the method, used in this investigation, to keep the amount of complement, which under the conditions of the test causes haemolysis of 50% of erythrocytes, so constant, as to make daily complement titration unnecessary.

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2. Dr A. BRANDWIJK, Director of the Routine Department of the State Institute of Public Health, Utrecht, who was so kind in allowing me to obtain sheep's blood from the stables of the Institute.

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# UEBER DIE GATTUNG *LAESTADIA* UND DIE GNOMONIACEEN

von

**J. A. VON ARX**

(Eingegangen am 12. Mai 1951).

Im Laufe von Untersuchungen, die die Revision einiger amerosporer Pyrenomycetengattungen zum Ziele hatten und über die an anderer Stelle berichtet werden soll, sind mir auch einige Formen begegnet, deren Ascosporen sich bei völliger Reife als mehrzellig erwiesen. Ein derartiger Fall liegt bei der Typusart der Gattung *Laestadia* Auersw. vor. Diese soll hier besprochen und gleichzeitig ihre Stellung im System erörtert werden.

Die Ascomycetengattung *Laestadia* hat AUERSWALD (1869*a*) in Unkenntnis einer bereits bestehenden Phanerogamengattung *Laestadia* Kunth (1832) aufgestellt. Als erste Art wird vom Autor *Sphaeria alnea* Fr. angeführt. Diese hat deshalb als Typus der Gattung zu gelten.

Wegen dem älteren Homonym *Laestadia* Kunth haben VIALA und RAVAZ (1892) den Namen *Guignardia* zur Bezeichnung der von ihnen besprochenen *Sphaeria Bidwellii* Ellis neu eingeführt. Ohne die Typusart von *Laestadia* Auersw. zu kennen, glaubten sie, ihn für alle bisher unter diesem Namen bekannten Arten anwenden zu können. Nun ist aber, wie mir der Vergleich von Originalexemplaren zeigte, *Sphaeria Bidwellii* ein von *Spaeria alnea* Fr. generisch völlig verschiedener Pilz. Hingegen stimmt die von AUERSWALD an zweiter Stelle angeführte *Laestadia punctoidea* (Cke.) Awd. mit *Guignardia Bidwellii* generisch überein. Dies ist auch von der Grosszahl der bis heute beschriebenen *Laestadia*- bzw. *Guignardia*-Arten der Fall. Ich glaube deshalb, mich der Ansicht v. HÖHNEL's (1918) anschliessen zu müssen, der *Sphaeria Bidwellii* Ellis, der einzigen von VIALA und RAVAZ bei der Aufstellung der Gattung *Guignardia* besprochenen Art, als deren Typus ansieht.

Von *Sphaeria alnea* Fr. als Typusart von *Laestadia* Auersw. hat v. Höhnel (1918) den Gnomoniaceencharakter erkannt. Ihm stand aber nur unreifes Material des Pilzes zur Verfügung; er stellte in der Folge für ihn die neue Gattung *Gnomonina* auf, die er wegen der scheinbar fehlenden Mündung bei den *Perisporiales* untergebracht wissen wollte.

Ich konnte nun mehrere Kollektionen dieses Pilzes untersuchen. Darunter befanden sich auch zwei Originalexemplare aus dem Herbarium Fries, die mir die Direktion des botanischen Museums in Uppsala in dankenswerter Weise zur Verfügung stellte. Bei den meisten Exemplaren war der Pilz noch unreif, nur bei wenigen konnte ich einige gut entwickelte Fruchtkörper mit reifen Sporen finden. Nach meinen Beobachtungen entwickelt sich dieser interessante Ascomycet folgendermassen:

Die Fruchtkörper wachsen auf den Blättern regelmässig zerstreut, längs den Blattnerven stehen sie häufig in unregelmässigen Reihen. Von oben gesehen sind sie mehr oder weniger rund, oft auch beinahe dreieckig mit abgerundeten Ecken. Am Rande, meist an der den Blattnerven abgekehrten Seite ist ein etwas hellerer, rundlicher Fleck von 30—40 $\mu$ Durchmesser zu sehen. An dieser Stelle besteht die Perithecienwand aus hellwandigeren Zellen. Vorerst ist der Fruchtkörper aber noch völlig geschlossen, erst später entwickelt sich von dieser Stelle aus das Ostiolum.

Im Querschnitt sind die Perithecien in der Richtung senkrecht zur Blattfläche etwas linsenförmig zusammengedrückt, oft eingesunken; sie entwickeln sich etwas unter der Epidermis und nehmen fast die ganze Blattdicke ein. Ihr Durchmesser beträgt 160—240 $\mu$, und sie erreichen eine Höhe von 100—160 $\mu$. Die Perithecienmembran besteht aus 3—5 Lagen von aussen ziemlich dickwandigen, dunkel-purpurbraunen, derben Zellen von 8—16 $\mu$ Grösse. Diese sind oben und unten ziemlich stark zusammengedrückt, an den Seiten aber fast polyedrisch; hier ist die Membran vor allem an der Stelle, wo später das Ostiolum angelegt wird, stark verdickt und besteht aus einem Parenchym von hellen, plasmareichen Zellen (siehe Fig. 1*a*). Mit der Weiterentwicklung beginnen sich von dieser Stelle aus erst die innern Parenchymzellen zu strecken und werden zu periphysenähnlichen, stark lichtbrechenden Fäden. Im hellen Fleck werden durch die von innen heranwachsenden Zellen die äussern gesprengt und die rund um diese Stelle stehenden beginnen ebenfalls in die Länge zu wachsen und sich zu teilen. So entsteht seitlich im Parenchym ein mit Periphysen ausgekleideter Kanal, der sich im schnell heranwachsenden und bei der Reife eine Länge vom mehrfachen Peritheciendurchmesser erreichenden Ostiolum fortsetzt. Dieses ist schnabelförmig; es besteht aus langgestreckten, fast faserigen, hyalinen oder schwach bräunlich gefärbten Zellen (Fig. 1*b*) und bricht meist blattunterseits hervor. Die Mündung entsteht also seitlich und biegt sich dann häufig noch innerhalb des eigentlichen Fruchtkörpers um 90° um.

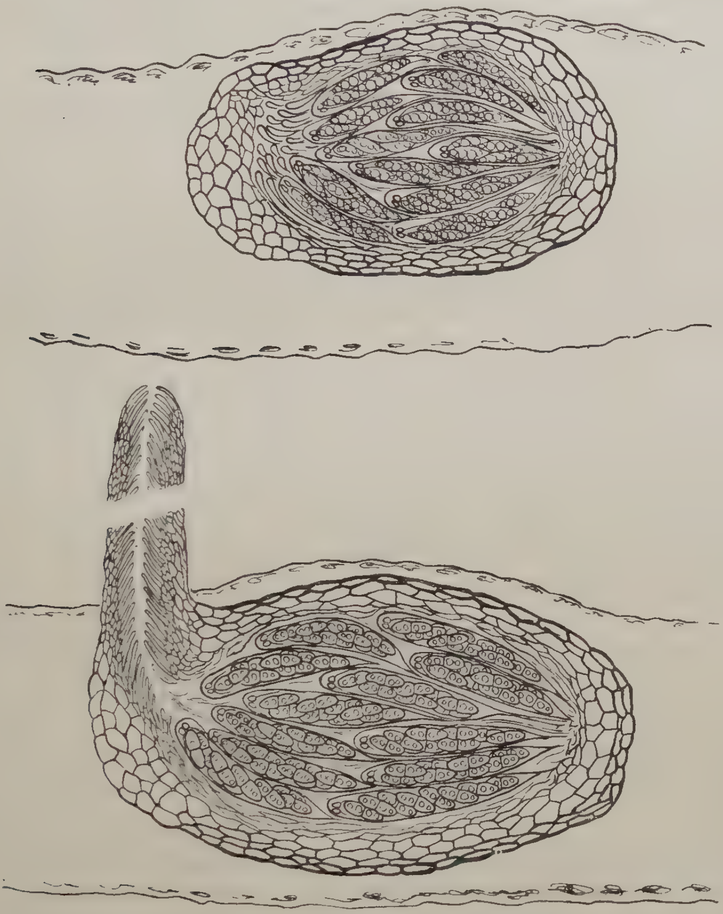

Fig. 1.

Medianschnitte durch Fruchtkörper von *Sphaeria alnea* Fr. (350 ×).
*a.* Unreifes, noch geschlossenes Perithecium.
*b.* Reifes Perithecium mit langem, seitenständigem Schnabel.

Die Asci sind wagrecht gelagert und mit ihrer Spitze gegen das Ostiolum gerichtet. Sie sind keulig-cylindrisch, oft etwas spindelig, unten in einen zarten, verschieden langen Stiel verschmälert, oben oft etwas verjüngt und abgerundet. Sie messen (50—70) × (9—12) μ; ihre Membran ist dünn und zart. Durch Verschleimen des Stieles kommen die Schläuche frei auf verscheidener Höhe zu liegen. In der Spitze ist die Ascusmembran verdickt; hier befindet sich der Apikalring, welcher im optischen Schnitt als 2 breit-keilförmige, eng aneinander gelehnte, stark licht brechende Körperchen von 3—4 μ Länge erscheint.

Die Sporen liegen zu 8 zweireihig im Ascus; sie sind hyalin, ellipsoidisch-spindelförmig, in der Mitte septiert und schwach eingeschnürt, beidends breit abgerundet und enthalten in jeder Zelle meist 2 Oeltröpfchen. Sie messen (16—20) × (4—6) μ. Paraphysen wurden nicht beobachtet.

Klebahn (1918) hat von diesem Pilz reifes Material untersucht und ihn als *Gnomonia Vleugelii* neu beschreiben. Nach Infektionsversuchen hat er ihn auf überwinterten Blättern im folgenden Frühsommer wieder erhalten. Er hat auch in Agarkulturen die Bildung von Perithecien mit reifen Asci und Sporen beobachten können.

Wie aus obiger Diagnose hervorgeht, ist *Sphaeria alnea* eine *Gnomonia* Ces. et de Not. mit liegenden, um 90° gedrehten Fruchtkörpern und scheinbar seitlich entspringenden Mündungen. Für derartige Formen hat bereits Fuckel (1869) die Gattung *Plagiostoma* begründet. Diese wurde von späteren Autoren, vor allem von Saccardo (1882) und Winter (1887) wieder mit *Gnomonia* vereinigt. Sie besteht aber, wie v. Höhnel (1918) gezeigt hat, mit Recht. Ihre Typusart *P. euphorbiae* Fuck. hat, wie die Untersuchung eines Fuckel'schen Exemplares zeigte, ebenfalls liegende, etwas eingesunkene Perithecien mit seitlich hervorbrechendem Schnabel. *Laestadia alnea* ist daher zu *Plagiostoma* zu stellen.

Ebenfalls eine Plagiostoma ist *Hypospila Rehmii* Sacc. (ausgegeben in Sydow, Mycoth. germ. no. 793). v. Höhnel (1918) hat den auf Blättern von *Carpinus Betulus* wachsenden Pilz zu *Gnomonina* gestellt.

Als Typus von *Hypospila* Fr. betrachtet v. Höhnel (1918) die von Fries (1849) bei der Begründung der Gattung an zweiter Stelle angeführte *H. bifrons* (D.C.) Fr. Dieser Pilz besitzt ein ausgebreitetes Pseudostroma mit epidermalem Klypeus; vor allem aber sind seine Ascosporen apiospor (= zweizellig, untere Zelle aber sehr klein oder höchstens einen Drittel der Länge der oberen erreichend).

*H. bifrons* (D.C.) Fr. wie auch die ebenfalls auf *Quercus*blättern wachsende, am besten als *Hypospila* Karsten bekannte *Sphaeria pustula* Pers. hat Klebahn (1918) eingehend beschrieben und abgebildet. Letztere Art kann aber nicht bei *Hypospila* bleiben, da sich bei ihren Sporen die Querwand in der Mitte befindet. Ferner fehlt das Pseudostroma fast völlig oder ist nur andeutungsweise durch eine schwache Braunfärbung des Substrates über den Perithecien wahr zu nehmen. Ein Klypeus ist ebenfalls nicht ausgebildet. Die Perithecien stehen meist einzeln, die derbe Epidermis der Eichenblätter wird durch sie beiderseits pustelförmig emporgewölbt. Wie bei *Sphaeria alnea* erscheinen die Fruchtkörper von oben gesehen rund, in Schnitten aber sind sie ellipsoidisch, also etwas platt gedrückt. Der Schnabel entspringt seitlich, vorerst verläuft er, oft gekrümmt, im Mesophyll des Blattes, um dann rechtwinklig umzubiegen und die Epidermis des Blattes meist hypophyll zu durchbrechen. Die Perithecienmembran ist ziemlich weich- und hellwandig; sie besteht aus 3—5 Lagen von stark zusammengepressten Zellen. Die Perithecien mitsamt den Hälsen sind von einer homogenen, ca. 10 $\mu$ dicken, hyalinen Schichte umgeben, die sich leicht ablöst. In dünnen Schnitten erhält man daher den Eindruck, als ob die Perithecien in einer Höhle liegen würden. Nach v. Höhnel (1918) besteht diese Schicht aus einer organischen Grundmasse, in der viel oxalsaurer Kalk kristallin eingelagert ist. Deshalb hat er den Pilz in die neue Gattung *Chalcosphaeria* gestellt.

Dem Baue nach ist *Sphaeria pustula* eine typische *Plagiostoma*. Die homogene Schicht, die die Perithecien umgibt, ist wohl für die Art charakteristisch; meiner Ansicht nach ist es aber abwegig, auf diesem rein äusserlichen und zudem leicht zu übersehenden Merkmal eine eigene Gattung zu begründen. *Sphaeria pustula* ist daher ebenfalls zu *Plagiostoma* zu stellen, wo *Chalcosphaeria* v. H. als Untergattung bestehen bleiben kann. Der Pilz stimmt auch trotz der gegenteiligen Ansicht von Winter (1887) mit *Plagiostoma subspecta* Fuck. volkommen überein und ist mit dieser identisch.

Nach dem gesagten ergibt sich für *Plagiostoma* und die neu zur Gattung zu stellenden Arten folgende Synonymie und Charakteristik:

*Plagiostoma* Fuck. Symb. myc. p. 118, 1869.

syn.: *Laestadia* Auersw. Hedwigia **8**, 177, 1869 (non Kunth 1832).
*Gnomonina* v. Höhn. Ann. myc. **16**, 46, 1918.
*Chalcosphaeria* v. Höhn. Ann. myc. **16**, 97, 1918.

Perithecien ohne deutliches Pseudostroma oder Klypeus dem Substrate vollkommen eingesenkt, liegend, von oben rundlich, im Medianschnitt abgeflacht, erst völlig geschlossen, früher oder später mit einem seitlich entspringenden und rechtwinklig umbiegend nach aussen mündenden, periphysierten Schnabel hervorbrechend. Asci dünnwandig, gestielt, auf verschiedener Höhe stehend, mit Apikalring. Die hyalinen Ascosporen sind ungefähr in der Mitte septiert.

Typus: *P. euphorbiae* Fuck. Symb. myc. p. 118, 1869.

Syn.: vide *Winter* (1887).
Matr.: *Euphorbia palustris*.

*P. pustula* (Pers.) comb. nov.

Syn.: *Sphaeria pustula* Pers. Syn. meth. Fung. p. 91, 1801.
*Phoma pustula* Fr. Syst. myc. **2**, 547, 1823.
*Sphaeria pleuronervia* de Not. Micromyc. ital. Dec. **9**, no. **9**, 1855.
*Isothea pustula* Berk. Outl. of Brit. Fungi p. 392, 1860.
*Sphaeria oleipara* Sollm. Hedwigia **4**, 65, 1865.
*Gnomonia pustula* Auersw. Mycol. europ. V/VI, p. 21, 1869.
*Sphaeria subspecta* Fuck. Fungi rhen. no. 2017.
*Plagiostoma subspecta* Fuck. Symb. myc. p. 119, 1869.
*Hypospila pustula* Karst. Mycol. fennica **2**, 127, 1873.
*Gnomonia subspecta* Sacc. Syll. fung. **1**, 566, 1882.
*Chalcosphaeria pustula* v. H. Ann. myc. **16**, 97, 1918.
Matr.: fol. *Quercus* spec.

*P. Rehmii* (Sacc.) comb. nov.

Syn.: *Hypospila Rehmii* Sacc. Syll. fung. **3**, 189, 1883.
*Gnomonina Rehmii* v. H. Ann. myc. **16**, 52, 1918.
Matr.: fol. *Carpinus Betulus*.

*P. alnea* (Fr.) comb. nov.

Syn.: *Sphaeria alnea* Fr. Syst. myc. **2**, 520, 1823.
*Sphaerella alnea* Auersw. Mycol. europ. V/VI, 1869.
*Laestadia alnea* Auersw. Hedwigia **8**, 177, 1869.
*Gnomonina alnea* v. H. Ann. myc. **16**, 47, 1918.
*Guignardia alnea* Schröt. Pilze Schlesiens II, p. 330, 1894.
*Gnomonia Vleugelii* Kleb. Ascom. p. 251, 1918.
*Plagiostoma Vleugelii* v. H. Hedwigia **62**, 49, 1921.
Matr.: fol. *Alnus* spec.

Die Gattung *Plagiostoma* gehört zu den Gnomoniaceen und unterscheidet sich von *Gnomonia*, wie schon gesagt, durch die liegenden Perithecien mit den seitlich hervorbrechenden und rechtwinklig nach oben biegenden Ostioli. Die einzelnen Gattungen der Familie unterscheiden sich neben diesem Merkmal durch die Zellenzahl und Form der Sporen. Die Familie ist folgendermassen zu charakterisieren:

*Gnomoniaceae.*

Perithecien einzeln stehend, ohne Stroma oder zu wenigen einem klypealen Pseudostroma eingebettet, immer dem Substrat eingewachsen, nur selten im Alter mehr oder weniger frei werdend. Jung sind die Fruchtkörper völlig geschlossen, öffnen sich aber meist früh an einer praedestinierten, oben oder scheinbar seitlich liegenden Stelle seltener mit einem periphysierten Porus, häufiger mit einem oft lang fadenförmigen, oft kürzeren, mehr oder weniger dunkel oder hell gefärbten, innen mit zahlreichen Periphysen ausgekleideten Schnabel. Die dünnwandigen, mit einem Apikelring versehenen Asci sind gestielt und stehen auf verschiedener Höhe; durch Verschleimen des Stieles liegen sie später frei in der Fruchtkörperhöhlung. Die Ascosporen sind hyalin, ein- oder mehrzellig oder fadenförmig. Paraphysen fehlen ganz oder sind fadenförmig, verschleimen aber meist bis zur Sporenreife.

Die Familie ist folgendermassen in Gattungen aufzuteilen:

Ascosporen einzellig, Asci 8 sporig.

> Perithecien klein, oben mit einem schwach entwickelten Klypeus, mit einem periphysischen Porus mündend (Schnabel fehlt völlig). . . . *Sphaerognomonia* Potebnia 1910.
>
> Perithecien klein, mit kurzem Schnabel, in einem ausgebreiteten Stroma sitzend. Flecken bildende Blattparasiten. *Diplacella* Syd. 1930.
>
> Perithecien aufrecht, ziemlich gross, mit einem lang schnabelförmigen Ostiolum hervorbrechend. Stroma fehlt . . . . . *Gnomoniella* Sacc. 1881.
>
> Perithecien ohne Stroma, hell und ziemlich dünnwandig in den Gehäusen grösserer Pyrenomyceten wachsend . . . *Cryptonectriopsis* v. Höhn. 1919.

Ascosporen ein- oder durch Pseudosepten scheinbar 2-zellig. Asci vielsporig . . . . . . . . . . . . . . . . . *Ditopella* de Not. 1863.

Ascosporen 2-zellig, untere Zelle sehr klein (apiospor).

Perithecien aufrecht, Schnabel oben entspringend . . . .
*Apiognomonia* v. H. 1917.

Perithecien liegend, mit seitlich entspringendem Ostiolum
Ohne Pseudostroma . . . *Plagiostomella* v. H. 1917.
Mit klypealem Pseudostroma . *Hypospila* Fr. 1849.

Ascosporen ungefähr in der Mitte septiert oder endlich vierzellig, oval bis lang-spindelförmig (Bei einigen Arten ist die obere Zelle etwas kleiner als die untere). Asci 8-sporig.

Perithecien aufrecht, Ostiolum oben eingesetzt . . . . .
*Gnomonia* Ces. et de Not. 1861.

Perithecien liegend, Ostiolum seitlich . . . . . . . . . . .
*Plagiostoma* Fuck 1869.

Ascosporen lang spindelförmig oder durch die starke Einschnürung bei der sich in der Mitte befindenden Querwand fast doppelspindelig, oft in 2 Teilstücke zerfallend. Perithecien liegend . . . . .
*Pleuroceras* Riess 1854.

Ascosporen schmal keulig, fast fadenförmig, im obern Drittel mit einer Querwand. Obere Zelle etwas angeschwollen, untere Zelle als schwanzartiges, fadenförmiges Anhängsel ausgebildet.

Perithecien aufrecht. . . . . *Ophiognomonia* Sacc. 1899.

Ascosporen fadenförmig, wurmförmig oder lang-cylindrisch, endlich mit Pseudosepten.

Perithecien liegend, in einem klypealen Pseudostroma eingebettet. Sporen dünn faden- oder nadelförmig . . . . .
*Linospora* Fuck. 1869.

Klypeales Stroma fehlt. Sporen cylindrisch oder wurmförmig.

Perithecien kugelig, aufrecht, mit einem oben oder etwas exzentrisch aufgesetzten Ostiolum . . . . . . . . . . .
*Gaeumannomyces* v. Arx et Olivier 1951.

Perithecien liegend, in der Substratrichtung gestreckt. Ostiolum seitlich, rechtwinklig aufwärts gebogen . . . .
*Plagiosphaera* Petr. 1941.

Ausgenommen die hier nicht anerkannten und die inzwischen neu hinzugekommenen Gattungen stimmt dieses System im

Wesentlichen mit dem von v. HÖHNEL (1917) entworfenen überein. Die *Diaporthaceen*, die inzwischen von WEHMEYER (1933) monographisch bearbeitet worden sind, müssen als eigene Familie aufgefasst werden und sind hier weggelassen. Da innerhalb der *Gnomoniaceen*-Gattungen vor allem nomenklatorisch Verwirrung und Unsicherheit herrscht, sollen die Typusarten noch kurz genannt und vor allem nach Literaturstudien besprochen werden.

1. *Sphaerognomonia carpinea* (Fr.) Pot. ist bis heute die einzige Art der von POTEBNIA (1910) aufgestellten Gattung. Eine gute Abbildung der Fruchtkörper findet sich bei KLEBAHN (1918). Die Gattung *Apiosporopsis* (Trav.) Mariani (1911) ist synonym und beruht auf einem Irrtum.

2. Von *Diplacella paullinae* (Frag. et Cif.) Syd. wurde nach einem Originalexemplar die beigegebene Abbildung entworfen. SYDOW (1930) hat den Pilz gut und ausführlich beschrieben. Er formt als Parasit typische Blattflecken, in denen das Gewebe fast volkommen durch das parenchymatische oder hyphige, lockere Pilzstroma verdrängt ist. *D. paullinae* ist eine der wenigen, tropischen Gnomoniaceen.

3. *Gnomoniella tubaeformis* (Tode) Saccardo (1881), die einzige sichere Art der Gattung hat KLEBAHN (1908) eingehend untersucht und abgebildet.

4. Als Typusart von *Cryptonectriopsis* v. H. hat der von REHM in Ascom. exs. no. 1523 als *Phomatospora ovalis* (Pass.) Sacc. ausgegebene Pilz zu gelten. v. HÖHNEL (1918) hat ihn wegen der hellen Perithecienmembran erst als *Hyponectria* aufgefasst und für ihn die neue Untergattung *Cryptonectriopsis* begründet; diese hat er 1919 zur Gattung erhoben. Leider gibt dieser Name eine ganz falsche Vorstellung über die Verwandtschaft des Pilzes, der mit den Nectriaceen nichts zu tun hat. Die Typusart *Cryptonectriopsis biparasitica* v. H. (syn. *Hyponectria biparasitica* v. H.) lebt saprophytisch in alten, entleerten Gehäusen einer *Leptosphaeria* (vgl. Fig. 3).

5. Mit *Ditopella ditopa* (Fr.) v. H. = *D. fusispora* de Not. ist *D. cryptosphaeria* (Fuck.) Sacc. identisch. Der Pilz wächst vor allem auf Zweigen, seltener auf Blattstielen von *Alnus*. Synonym zur Gattung ist *Rehmiella* Winter (1883).

6. Als Typusart von *Apiognomonia* bezeichnet v. HÖHNEL (1917, 1918) *A. veneta* (Sacc. et Speg.) v. H. Dieser Pilz wächst auf Blättern von *Platanus*. Auf andern Substraten, z.B. auf Blättern von *Quercus*, *Fagus* und *Tilia* kommen nun Pilze vor, die morphologisch

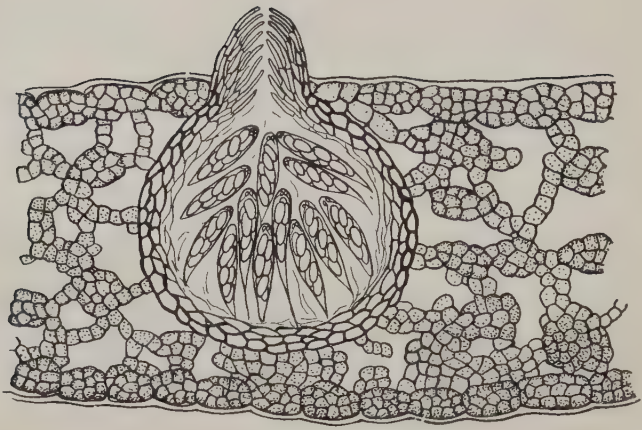

Fig. 2.
Querschnitt durch eine Stromapartie von *Diplacella paullinae*, mit einem Perithecium (350 ×).

mit *A. veneta* vollkommen übereinstimmen und sich spezifisch nicht voneinander trennen lassen. Am frühesten wurde dieser Pilz als *Sphaeria errabunda* Rob. beschrieben. Für die Typusart ergibt sich daher folgende Synonymie:

*Apiognomonia errabunda* (Rob.) v. H. Ann. myc. **16**, 51, 1918.

Syn.: *Sphaeria errabunda* Rob. in Desmazières, Ann. sc. nat. 3 sér. **10**, 355, 1848.
*Gnomonia errabunda* Auersw. Mycol. europ. V/VI, p. 25, 1869.
*Laestadia veneta* Sacc. et Speg. Michelia **1**, 351, 1881.
*Laestadia errabunda* Rehm
*Laestadia errabunda* Rehm f. tiliae Rehm. Ascom. exs. no. 1426.
*Gnomonia veneta* [1]) Kleb. Jahrb. wiss. Bot. **41**, 533, 1905.
*Apiosporopsis veneta* Mariani Atti soc. ital. sc. nat. **50**, 165, 1911
*Gnomonia platani* Kleb. 1914 (zit. Ascom. p. 59, 1918).
*Gnomonia quercina* Kleb. Ascom. p. 178, 1918.
*Gnomonia tiliae* Kleb. Ascom. p. 202, 1918.
*Apiognomonia veneta* v. H. Ann. myc. **16**, 51, 1918.
*Apiognomonia quercina* v. H. Hedwigia **62**, 48, 1921.
*Apiognomonia tiliae* v. H. Hedwigia **62**, 48, 1921.

Dieser parasitisch auf verschiedenen Pflanzen lebende Pilz bildet auf denselben Substraten eine übereinstimmend gebaute Konidien-form, welche von KLEBAHN (1905, 1918) als *Gloeosporium* beschrieben und abgebildet und von v. HÖHNEL (1921) wegen dem intraepidermalen Wachstum zu *Myxosporina* gestellt wurde.

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[1]) *Gnomonia veneta* Speg. ist verschieden und eine gute Art der Gattung; bei den Ascosporen dieses Pilzes ist die obere Zelle etwas kleiner als die untere.

7. Als Typusart von *Plagiostomella* hat nach v. HÖHNEL (1919, 1921) *P. carpinicola* v. H. zu gelten, vom Autor 1918 auf Grund des in REHM, Ascom. exs. no. 496 und 1425 fälschlicherweise als *Apiospora carpinea* (Fr.) Rehm ausgegebenen Materials beschrieben. Die 1917 und 1918 als Typusart angegebene *Plagiostoma petiolicola* Fuck. ist nach der Abbildung bei KLEBAHN (1918) eine gute *Plagiostoma*. Bei den Sporen dieses Pilzes ist die obere Zelle gegenüber der untern etwas kürzer und schmäler; also sind sie nicht apiospor.

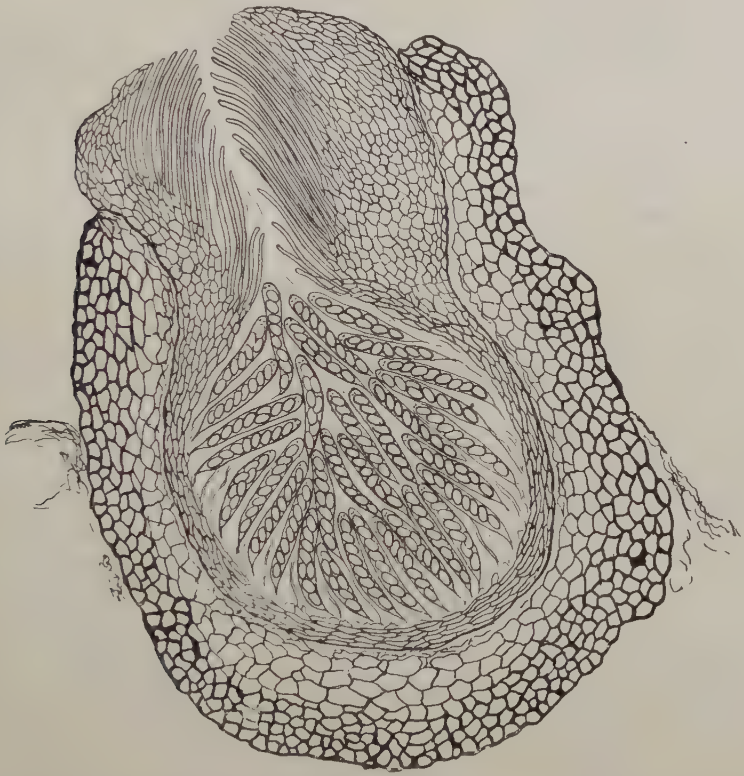

Fig. 3.

Querschnitt durch einen in einem alten Perithecium schmarotzenden Fruchtkörper von *Cryptonectriopsis biparasitica* (350 ×).

8. *Hypospila* Fries (syn. *Hypospilina* (Sacc.) Trav.) und *Plagiostoma* Fuck. siehe oben.

9. *Gnomonia* Ces. et de Not. mit *G. setacea* (Pers.) de Not. als Typusart ist die artenreichste Gattung der Familie. Die Sporen sind oval bis lang spindelförmig und ungefähr in der Mitte septiert; bei einigen Arten zeichnen sie sich durch kurze, spitze Anhängsel aus. Bei vielen *Gnomonia*- (wie auch *Plagiostoma*-)arten enthalten die Sporen 4 Oeltropfen, was auf eine spätere Vierzelligkeit hinweist. Für Arten mit endlich 4-zelligen Sporen besteht die Gattung *Gnomoniopsis* (Winter) Berlese, die sich aber nur schwierig von *Gnomonia* abtrennen lässt.

10. *Pleuroceras* Riess (1854) ist der ältere Name für *Cryptoderis* Auerswald (1869*b*). Die beiden Gattungen haben denselben Pilz zur Typusart; dieser hat *Pleuroceras cryptoderis* (Lév.) v. H. zu heissen (vgl. v. Höhnel, 1918).

11. *Ophiognomonia melanostyla* (D.C.) Sacc. ist die einzige, sichere Art der von Saccardo (1899) zur Gattung erhobenen Untergattung von *Gnomonia*. Dieser Pilz wurde von Klebahn (1918) ebenfalls eingehend untersucht und abgebildet.

12. *Linospora capreae* (D.C.) Fuck. wurde von Fuckel (1869) beim Aufstellen der Gattung erst an zweiter Stelle angefürt. An erster Stelle steht *L. candida* Fuck., welche mit *Cryptoderis lamprotheca* (Desm.) Auersw. = *Pleuroceras ciliatum* Riess = *P. cryptoderis* (Lév.) v. H. identisch ist. So muss die zweite Art als Typus gelten; sie entspricht auch der von Fuckel gegebenen Gattungsdiagnose. v. Höhnel (1918) nannte die bisher als *Linospora* bekannten Arten *Phoma*, weil die bei Fries (1823) unter dieser Gattung an erster Stelle angeführte *Phoma saligna* Fr. ein unreifes Stadium von *Linospora capreae* ist (vgl. auch Theissen und Sydow, 1917, p. 392).

*Ceuthocarpon* Karsten (1873) mit der Typusart *C. populinum* (Pers.) Karst. stimmt mit *Linospora* weitgehend überein und wird am besten mit dieser vereinigt. Im Stromabau finden sich keine wesentlichen Unterschiede. v. Höhnel (1917, 1918) glaubt, die Gattung sei durch die nicht vorragenden Mündungen gegenüber *Linospora* genügend charakterisiert.

13. *Gaeumannomyces* v. Arx et Olivier (1951) wurde für den bisher als *Ophiobolus graminis* (Sacc.) Sacc. bekannten Ascomyceten aufgestellt.

14. *Plagiosphaera Petrak* (1941) hat einen vom selben Autor erst als *Ophiobolus moravicus* beschriebenen Pilz zum Typus. Die zwei letztgenannten Gattungen sind bisher monotypisch.

In seinem System der Diaporthaceen erwähnt v. HÖHNEL (1917) auch die Gattungen *Mamiania* Ces. et de Not. (1863) und *Mamianiella* v. H. (1917, 1918). Beide unterscheiden sich nur in den Sporen, *Mamiania* ist apiospor, *Mamianiella* amerospor. Diese Pilze entwickeln sich auf lebenden Blättern, wo sie intramatrikal ein mächtiges Stroma bilden. Dieses ist nach aussen von einer dunklen, kohligen Kruste begrenzt, während das Binnengeflecht aus hyalinen, fleischigen Zellen bzw. Hyphen besteht; hypertrophisch durch ein enges Verwachsen von Pilzhyphen und Wirtszellen entstanden. In diesem hyalinen Kerngewebe werden die erst hell, später dunkelwandigen Perithecien angelegt, die dann mit einem dicken und ziemlich langen Schnabel hervorbrechen.

*Mamiania* und *Mamianiella* unterscheiden sich von den typischen Gnomoniaceen durch das grosse, sklerotiale, innen weisse Stroma; diese Gattungen sind zusammen mit *Mazzantia* Mont. und verwandten Formen in eine eigene Familie zu stellen.

Als weitere Familien der *Diaporthales* sind neben den Gnomoniaceen die Diaporthaceen (*Diaporthopsis* Fabre, *Cryptodiaporthe* Petr., *Diaporthe* Nit., *Chorostate* (Nit.) Trav., *Caudospora* Starb., *Endothia* Fr., *Hercospora* Tul., *Melanconis* Tul., *Diaporthella* Petr., *Apioporthe* v. H., *Apioporthella* Petr., *Prosthecium* Fres., *Vialaea* Sacc. und andere Gattungen), die Valsaceen [*Valsa* Fr. (Syn.: *Leucostoma* (Nit.) v. H., *Valsella* Fuck.), *Cryptospora* Tul.] und die Phomatosporaceen zu unterscheiden. Letztgenannte Familie umfasst Formen mit nicht oder nur kurz gestielten, mehr cylindrischen, mehr oder weniger parallel nebeneinander und auf gleicher Höhe stehenden Asci. Neben *Phomatospora* Sacc. gehören u.a. die Gattungen *Phomatosporopsis* Petr., *Ceratostomella* Sacc., *Sydowiella* Petr. und *Paradidymella* Petr. hierher.

Die Diaporthaceen und die Gnomoniaceen sind nahe miteinander verwandt und durch zahlreiche Uebergänge verbunden. Die letzteren umfassen eigentlich nur die kleineren, meist blattbewohnenden Formen der ersteren, die auf Aesten und auf Stengeln grösserer Kräuter leben und häufig ein kräftiges Stroma ausbilden.

## Summary.

1. The genus *Gnomonina* was described by v. HÖHNEL (1918) to accomodate *Sphaeria alnea* Fr., the type of the genus *Laestadia* Auersw. (non Kunth); he described it as a member of the *Gnomo-*

*niaceae* with completely closed fruiting-bodies. But when ripe these fructifications, which are buried in the substratum, lying parallel to the surface, develop an apparently lateral perithecial beak which erupts to the exterior. Ripe ascospores are two-celled. The fungus is, thus, a typical member of the genus *Plagiostoma* Fuck. and must be named *P. alnea* (Fr.) comb. nov.

2. *Sphaeria pustula* Pers. = *Hypospila* Karst. = *Chalcosphaeria* v. Höhn. is distinguished by the presence of a hyaline, apparently crystalline envelope, but agrees in all other structural details with the genus *Plagiostoma* and is identical with *P. subspecta* Fuck. It must be named *P. pustula* (Pers.) comb. nov.

3. *Hypospila Rehmii* Sacc. is also a *Plagiostoma*.

4. A general description of the family *Gnomoniaceae* and a key of the genera is given; the nomenclature of the genera and their type-species is briefly discussed.

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# BOOKS.

C. van Duin. Inleiding tot de microscopische techniek (Introduction to the microscopic technique, with a preface by Prof. Dr J. Boeke). N.V. Uitgevers-Maatschappij Æ. E. Kluwer, Deventer-Djakarta. 1950.

This book is intended to be a general guide for all persons using the microscope professionally: students in biology, pharmacy, medicine, microbiology and biochemistry. The book gives quite a lot of information on the microscope and its use in research work in these scientific branches. Without any doubt the author has been very succesful in fullfilling his task. Paying regard to the fact that it is hardly possible to be up to the level of all the fields of sciences mentioned above, it is no wonder that here and there omissions may be found. I am sure, that some criticism will reach him, which he may make use of in a second edition. W.

Leerboek der Microbiologie en Immunologie; onder redactie van A. Charlotte Ruys (textbook of Microbiology and Immunology; edited by A. Charlotte Ruys). N.V. A. Oosthoek's Uitgevers Mij., Utrecht. 1950.

This textbook of microbiology and immunology for practitioners and medical students is divided into a general and a special section. In the general section, which, considering the aim of the book, is fairly detailed, T. Y. Kingma Boltjes treats the form and function of the bacteria; J. Bijlmer the general biology of protozoa; J. D. Verlinde the general virology; A. B. F. A. Pondman infection and immunology; J. J. van Loghem the relation between the micro-organism and its host and K. C. Winkler the disinfectants, the chemotherapeutics and antibiotics.

In the more concise special section, A. Ch. Ruys deals with the bacteria, the pathogenic *Bacillaceae* excepted, which are treated by Pondman, H. Esseveld deals with the pathogenic fungi, Bijlmer with the pathogenic protozoa and Verlinde with the rickettsiae and the viruses.

Although the composition of a textbook by a number of experts may act unfavourably on its final unity, the Editor has rightly decided upon the following of this course. No single scientist is any longer able to have a sound knowledge of the whole field of microbiology. The presentation of the subject-matter is well balanced, the various problems in microbiology are discussed; as to some of them, not all bacteriologists will agree with the view expressed by the author in question.

An important feature for the general practitioners is the technical supplement by Ruys, in which she gives many advices concerning the collecting and the mailing of the material to be examined. The importance thereof for the reliability of the results cannot be overstressed.

Doubtless this Netherlands textbook will take its stand honourably among the many microbiological textbooks issued more recently in various countries and it will certainly be eagerly received by the Netherlands speaking workers in medical microbiology. J. H. BEKKER.

L. E. DEN DOOREN DE JONG. Microben als vrienden en vijanden van de mens (Micro-organisms as friends and ennemies of humanity). Vol. I. Uitgeverij H. Nelissen, Bilthoven. 1951.

The first volume contains as section 1: Basal facts of microbiology and Section 2: Micro-organisms as enemies of humanity. The second volume: will contain Section 3: The combating of micro-organisms and Section 4 Micro-organisms as friends of humanity.

The starting-point chosen in composing this book can best be described in the author's own words, cited from the Preface: ,,As an infection by micro-organisms is a mineralization by means of a violating action on living matter, there is no essential difference between the function of the innocent (saprophytic) and the disease inducing (pathogenic) micro-organisms. Therefore it has been aimed at to insert disease inducing micro-organisms, more especially the disease inducing bacteria, in the vast whole of the microcosmos''.

The author began his bacteriological career as a pupil of BEIJERINCK and his biological outlook has been greatly influenced by the creative imagination of his eminent teacher. After professional work in the food inspection service he occupies since many years a leading position as a medical bacteriologist. He thus disposes of a wealth of information and often presents this information by means of highly descriptive images. This will appeal to the general reader who, when looking for information in a for him unknown field, is generally discouraged by the use of mainly technical terms.

The interest of the reader is chiefly focused on the variety in ways and means of attack of the ,,inimical'' micro-organisms and the resulting ways and means of combating them. Symptoms of disease are passed over or treated very summarily and so the field of action of the physican is pointed at, but not entered upon. The value of the new tools in combating infectious diseases is stressed and in the referent's opinion it is mainly due to the existence of these that a book of this scope is desirable. Moreover the intricacy of medical bacteriology is sufficiently brought forward to impress the non-medical reader and keep him from using the book as a private vademecum.

In his preface and ,,apology'' the author mentions that next to the general reader the book is written in view of students in medicine, biology, pharmacy, agriculture and chemical technology in so far that it might supplement their regular scientific training. And next to these it might serve as a source of information for medical and chemical analysts, working in industrial or in controlling laboratories.

It seems to the non-medical referent that sometimes a too high standard of completeness is aimed at, but it seems to her as well, that a large and very varying group of readers may derive profit from the wealth of critically selected facts gathered in this volume. MARIE P. LÖHNIS.

# MAURICE LANGERON 1874—1950

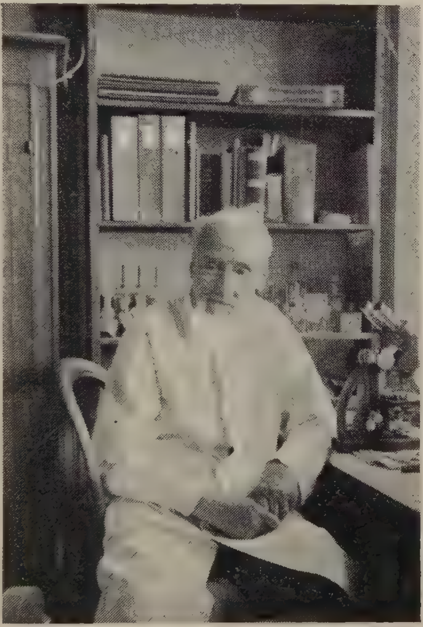
par

**JOHANNA WESTERDIJK et JACOMINA LODDER**

(Reçu le 26 Juin 1951).

Le décès de ce grand mycologue nous oblige à passer en revue sa vie et son œuvre.

Il commença ses études de médecine à Dijon, sa ville natale, il les poursuivit à Paris, où il consacra entre autres 4 ans à l'étude des plantes fossiles.



Après avoir terminé sa thèse, il se rendit en Allemagne pour apprendre l'allemand et, en même temps, pour étudier les mousses. Lorsqu'il fut retourné à Paris, on chercha en vain une place pour lui au Musée d'Histoire naturelle et cet homme si doué finit seulement par trouver une place fort modeste comme secrétaire du directeur de l'Institut de Parasitologie de la Faculté de Médecine aux appointements magnifiques de 50 francs par mois! Il ne pouvait gagner sa vie qu'en donnant au surplus des leçons de français aux étrangers. Son chef, BLANCHARD, l'initia à la mycologie et lui conseilla de rédiger la revue ,,Archives de Parasitologie''. Après la mort de BLANCHARD, BRUMPT lui succéda, celui-ci conseilla à LANGERON de voyager dans plusieurs pays de l'Amérique du Sud où il trouva le paradis des dermatophytes, qui l'avaient toujours intéressé.

Le professeur BRUMPT lui confia également la direction d'une section de mycologie médicale, faisant partie de l'Institut de Parasitologie. LANGERON donna chaque année des cours de mycologie et il fit plusieurs démonstrations pratiques, auxquelles assista une foule de médecins et de mycologues, de toutes les parties du monde, ses élèves se recrutaient donc parmi les nationalités les plus diverses. Tous les travaux pratiques qu'il dirigea, l'ont amené à composer son ,,Précis de Mycologie'', chef-d'œuvre paru en 1945, plein de remarques originales et de considérations spirituelles, que les botanistes, les mycologues et aussi les médecins dermatologues ont beaucoup étudié.

Quoiqu'il ait dit dans son avertissement: ,,Je n'ai jamais eu de prédilection pour les champignons, ou m'a conseillé l'étude de ce groupe. Quoiqu'ils n'aient pas ému ma sympathie, ils ont du moins forcé mon admiration, ces spécialistes des armes chimiques''. Nous vous disons: quiconque eut le bonheur de voir travailler Langeron avec ces êtres non-aimés et a entendu ses explications très enthousiastes et même très sympathiques, a bien eu l'impression que cette phrase de l'avertissement n'était que de la blague.

Qui n'a pas savouré son opinion: ,,le milieu synthétique est détestable, c'est un reliquat de cette chimie des corps purs, qui sévit encore dans l'enseignement, car toute la chimie élémentaire classique repose sur la notion illusoire du corps pur''.

Outre cet excellent ouvrage original, Langeron a reclassé complètement les dermatophytes sur une base vraiment botanique, selon les règles de nomenclature internationales. Une foule de descriptions nouvelles et d'images en sont le résultat. Il a fondé ses idées sur les teignes en les étudiant dans des milieux naturels divers, ce que ne firent pas les autres mycologues. Il étudia surtout le Trichophyton, l'Epidermophyton, les Sabouraudites et les Ctenomyces.

Des élèves de maints pays l'ont aidé à cette tâche énorme. Cependant le terrain des recherches et des publications de Langeron est plus vaste encore. Nous croyons pourtant qu'il portait un intérêt particulier et intense aux champignons levuriformes.

Avant les recherches de Langeron la classification des levures anascosporées filamenteuses, nommées à tort *Monilia* dans la littérature médicale, se trouvait dans un état désordonné. Le nombre des espèces décrites et des noms publiés était extrèmement grand, mais il était à peu près impossible d'identifier une souche nouvellement isolée.

Sur ce sujet parut en 1932 la première publication de Langeron en collaboration avec Talice. Non seulement Langeron s'était renseigné très à fond sur de nombreuses publications, mais il avait aussi étudié ces organismes d'une façon très personnelle. Il classa les levures anascosporées filamenteuses en 6 genres qui diffèrent surtout à cause de leurs caractères morphologiques: notamment la formation du pseudomycelium et la façon dont se développent les blastospores aux certicles du pseudomycelium.

Tandis qu'autrefois on étudiait presque exclusivement les caractères biochimiques, grâce à cette publication on connaît maintenant aussi la morphologie de ces levures.

Pourtant il parut qu'au point de vue morphologique ces levures étaient trop variables pour permettre de maintenir la classification en 6 genres et ce fut Langeron lui-même qui s'en aperçut le premier. En 1938 il publia en collaboration avec Guerra, une étude détaillée sur ce sujet, dans laquelle il abandonna cette classification. Il décrivit 16 espèces bien reconnaissables, toutes réunies dans un seul genre: *Candida.*

La publication de 1932 garde néanmoins son intérêt, parceque les genres décrits suivant leurs caractères morphologiques fournissent encore un point d'appui souvent appréciable pour la description des espèces aujourd'hui connues.

Les listes des synonymes appartenant à la publication de 1938 ont beaucoup de valeur, parce qu'elles établissent un rapport entre les espèces autrefois décrites et celles qui ont été reconnues par lui. Nous devons en premier lieu à Langeron de pouvoir identifier à présent une souche du genre *Candida.*

Le terrain de la mycologie ne lui suffit pas. Son ,,Précis de Microscopie'' (éd. 1942 et 1949), livre de 1396 pages, contenant toutes les techniques possibles de la botanique, de la zoölogie, de la microbiologie et de l'optique, nous le montre pleinement.

On ferait tort à Langeron de parler uniquement de ses mérites scientifiques. Son enthousiasme pour le travail, le vif intérêt qu'il portait aux recherches des autres, son extrême amabilité et son souci de rendre service ont fait une profonde impression sur nous toutes les fois que nous avons eu le privilège de le rencontrer ou de correspondre avec lui.

Nous perdons en lui non seulement un esprit original, un maître plein d'expérience, mais aussi un ami dévoué et très bon.

(Centraal Bureau voor Schimmelcultures, Baarn, Holland).

# *CANDELABRUM SPINULOSUM* A NEW FUNGUS SPECIES

by

**AGATHE L. VAN BEVERWIJK**

(Received May 26, 1951).

In the course of an investigation of helicosporous Fungi imperfecti growing on decaying leaves in water, the fungus which is the subject of this paper, was frequently observed.

Decaying plant material was collected from near the surface of ponds and other natural waters. During transport to the laboratory most of the spores of fungi growing on the leaves fall off, so the material is kept in Petri dishes with enough water to keep it moist for a few days, when new conidia develop. With the aid of a dissecting microscope a rich flora of fungi belonging to different groups of Hyphomycetes can be observed growing on the decaying leaves. Amongst these, the conidia of the fungus under consideration appear like sparkling white, minute rings, lying singly or more rarely in groups of two to three. The mycelium is not visible in reflected light. When the water in the Petri dish overflows the leaf, the conidia are readily detached from their conidiophores and may float in groups on the surface of the water. The conidia of this fungus have a very curious shape. Their appearance as seen from the top is strikingly different from their side view. When seen from above the conidium shows eight roundish cells decorated with blunt spines (fig. 1, *a*). When focussing slightly deeper these eight cells appear to be connected by an H shaped body consisting of 4 cells, the central cells (fig. 1, *b* and *c*).

Three septa divide the H shaped body into two larger and two smaller cells. The larger and the smaller cells may lie at the same side of the middle bar or may be in opposition (fig. 2, *f*).

The four central cells each bear two lateral cells, growing out at right angles to the central cells. This is seen more clearly in side

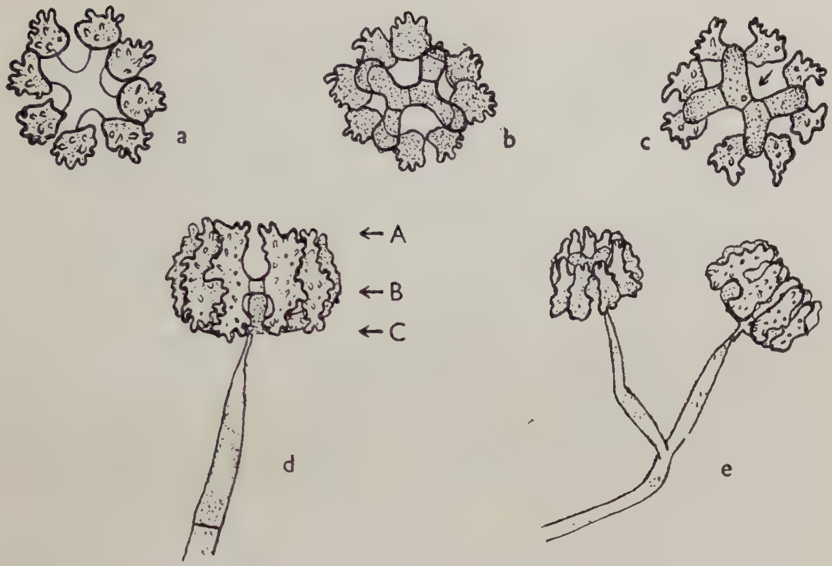


Fig. 1. (*a-d*: 1500×; *e*: 1100×).

*a.* conidium seen from above, the tops of the lateral cells in focus.
*b.* as *a*, but with lower focussing. Central cells with the pairs of lateral cells attached to them.
*c.* Conidium, seen from below, the scar left by the conidiophore is visible (indicated by arrow) on one of the central cells.
*d.* Conidium, side view. The arrows A, B and C indicate the levels of drawings *a*, *b* and *c*.
*e.* Branched conidiophore with two terminal conidia.
*a-d.* from culture material. *e* from leaf of *Castanea vesca*.

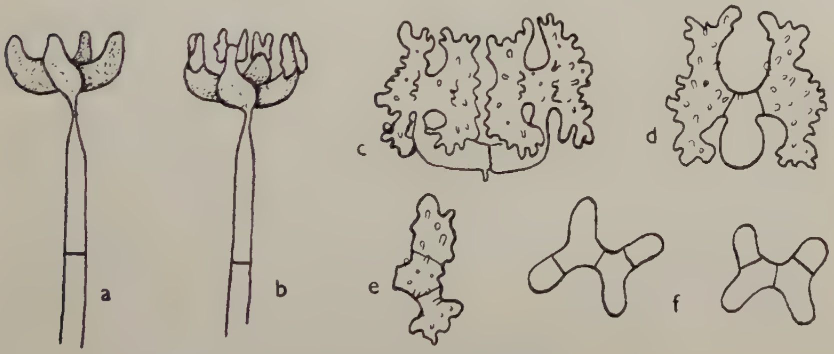


Fig. 2. Slightly diagrammatic.

*a.* the four central cells (side view).
*b.* the lateral cells are beginning to develop on the apices of the central cells (side view).
*c.* two pairs of lateral cells with their central cells.
*d.* one pair of lateral cells on apex of a central cell.
*e.* one lateral cell with cross walls.
*f.* the four central cells, showing different arrangement of septa.

view (fig. 2, *b*, *c* and *d*). Each pair of lateral cells is attached a little below the middle to the apex of a central cell which is slightly bent upwards. Cross walls develop between the lateral cells and the central cell (fig. 2, *d*). The eight lateral cells have an oblong irregular shape. They are indented to lobed and densely covered with blunt spines which gives them an almost fringed appearance. The spines do not stain with cotton blue. Very rarely septa are seen in the lateral cells, their lobed appearance and dense spines, however, make observation of details difficult (fig. 2, *e*). The conidium is more or less cup shaped, the central cells forming the bottom, the lateral cells the wall, their upper ends (the eight cells that were seen first in the high focussing) are the rim of the cup. Or, expressed more precisely, the conidium reminds one of a four-armed chandelier, each arm holding two elaborately decorated candle-sticks to the left and the right of its free end (fig. 1, *d* and *e*).

The spore measurements are: in top view: diameter of conidium 14 (12—15) μ, apex of lateral cell 2—2.5 μ. The difference in focussing between the upper end of the lateral cells and the central cells is about 3—4 μ. The four central cells (measured diagonally) 8.5—11 μ. In side view: 12—15 × 7.5—10 μ. The width of the lateral cells varies from 2 to 4 μ because of the irregular shape of the cell.

Necol mounts (Ellis (2)) of dried leaves on which the fungus was found growing, show the mycelium and conidiophores. The mycelium branches in the debris on the leaf, the erect conidiophores grow through the water film on the leaf and develop conidia just above the water surface. For this type of growth: vegetative mycelium under water (or in the water film on plant material) producing conidiophores which develop conidia just above the water surface, the term aero-aquatic fungus has been suggested (van Beverwijk (1)).

The length of the conidiophore varies with the depth of the waterfilm it has to pierce. Conidiophores of 18—55 μ were measured.

The conidiophore is a single side branch of the mycelium, forming a terminal conidium, occasionally it is branched and bears two or three conidia. The width of the conidiophore is 2—3 μ, the apical cell tapers to a sharp point (fig. 1, *d* and *e*).

The following strains were collected:

| locality | growing on | time of the year |
|---|---|---|
| pond near Ommen, Netherlands | decaying leaves of *Betula* | March and July 1949 |
| brook near Minusio, Tessin, Switzerland | twig (not identified) and leaf of *Castanea vesca* | October 1949 |
| ponds in wood near Baarn, Netherlands | leaf of *Carpinus betulus* and *Quercus* | March and October 1949 |
| pond in Drottningholm (Stockholm), Sweden | *Alnus* leaf and female catkin | July 1950 |
| brook near Houthem, Netherlands | different decaying leaves | September 1950 |
| old bombcrater partly filled with rainwater, Baarn, Netherlands | leaf of *Betula* | October 1950 |

From this list it may be gathered that *Candelabrum spinulosum* is a wide spread fungus and that it may be found practically throughout the year on various types of decaying plant material in water.

Several strains of the fungus have been isolated in pure culture. Under a dissecting microscope spores are picked off from the plant material with the aid of a fine glass needle and transferred to cherry agar or to a hanging drop of cherry decoction.

The conidia germinate readily, sending out several germ tubes. Lateral cells as well as central cells are capable of producing one to several germ tubes (fig. 3). Good growth was obtained on most of the ordinary culture media, but it proved rather difficult to induce spore development. On and near the inoculum the vegetative mycelium has a plumose growth ("tufts of mycelium with a central axis from which short hyphae radiate", according to the definition of LONG and HARSCH (3)). It is cream coloured on most media. On 2 % malt agar the plumose growth of the center is surrounded by a zone of submerged mycelium. On carrot plug many narrow folds in the mycelial mat developed. No conidia were observed on these media.

On sterilized lupine stem some tufts of "woolly" (LONG and HARSCH (3)) mycelium develop near the inoculum and on the top of the lupine stem. Spore development is fair but not abundant.

The best fructification was obtained on Lutz agar:

| | |
|---|---|
| beech wood shavings | 175 g |
| water | 750 g |

Autoclave for 2 hours at 120° C., squeeze and filter. To 500 ml of this decoction add:

| | |
|---|---|
| xylose | 2.5 g |
| maltose | 2.5 g |
| ammonium phosphate | 0.5 g |
| ammonium nitrate | 0.5 g |
| agar | 10 g |

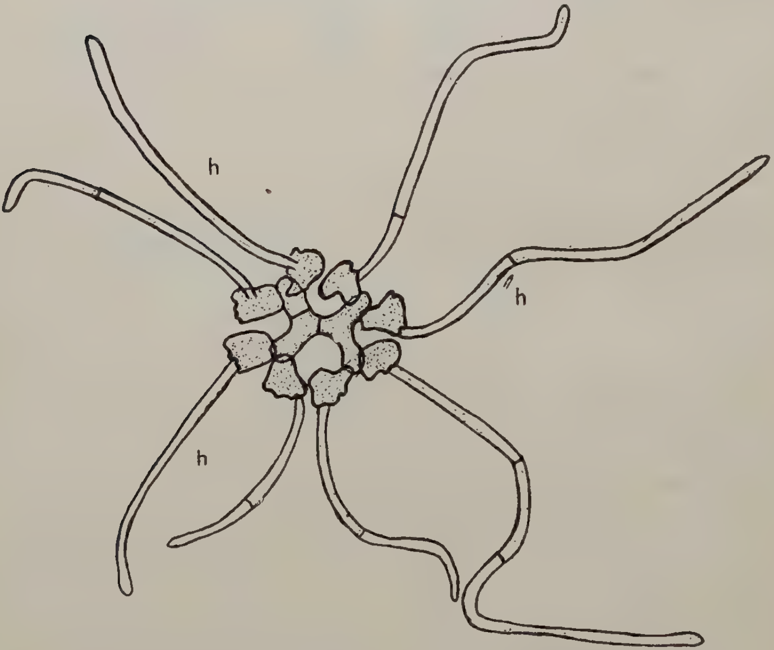


Fig. 3. Germinating conidium (1500 ×).
Each lateral cell has sent out one germ tube. The three hyphae marked *h* were only seen in top focussing. Germination after 20 hours in cherry decoction.

Generally, spores develop rather late on the sparse mycelium in the drying out upper part of the agar slant. Study of the optimum conditions for spore development is still in progress. Temperature (from 28° to 5° C.) seems to have little or no influence on sporing.

Recently an abundant spore development was obtained in some cultures on Lutz agar exposed to sunlight. The conidia develop on the pure white aerial mycelium. The growth of this sporing mycelium is woolly to felty, no conidia were observed in the plumose cream coloured mycelium of the same cultures. In culture as on plant material the conidia are readily shed, even in Necol mounts, as the point of attachment to the conidiophore is very narrow. Sometimes a small "stalk", 1—2 μ long, remains visible on one of the central cells (fig. 2, *c*). In plate and slide cultures vegetative growth was good but no conidia developed. No perfect stage has been observed.

No description of this curious fungus has been found in the literature and apparently no genus exists in which it may be placed. So a new genus, *Candelabrum*, is suggested, to contain the single species *Candelabrum spinulosum*.

### *Candelabrum* gen. nov.

Fungus aero-aquaticus mycelio hyalino, septato, ramoso. Conidiophora rami mycelii, conidia supra aquam formata. Conidia hyalina, ex pluribus cellulis constantia, quattuor cellulis in medio positis singulis binas cellulas laterales in apicibus gerentibus. Cellulae laterales oriuntur rectis angulis factis e cellulis in medio positis.

### *Candelabrum spinulosum* sp. nov.

Fungus aero-aquaticus mycelio hyalino, septato, ramoso. Conidiophora vulgo simplicia, in terminis gerentia singula conidia supra aquam formata. Conidia constant e quattuor cellulis in medio positis, quae in formam litterae H coniunctae sunt, et octo cellulis lateralibus. Cellulae laterales a laeva dextraque parte ex apicibus cellularum in medio positarum oriuntur rectis angulis factis cum ex directione, qua cellulae in medio positae crescunt. Cellulae in medio positae leves, cellulae laterales densis spinis hebetibus contectae. Conidia de super 14 (12—15) μ, a latere 14 (12—15) × 7.5 — 10 μ lata.

In membris plantarum sub aqua putrescentibus.

### *Candelabrum* n. gen.

Aero-aquatic fungus with hyaline septate branched mycelium.

Conidiophore a branch of the mycelium, conidia produced above the surface of the water.

Conidium hyaline, pluricellular, four central cells bearing each two lateral cells at their apices. The direction of growth of the lateral cells is perpendicular to that of the central cells.

### *Candelabrum spinulosum* n. sp.

Aero-aquatic fungus with hyaline septate branched mycelium.

Conidiophore usually simple with a single terminal conidium formed above the surface of the water.

Conidium consisting of an H shaped body of four central cells, and eight lateral cells. The lateral cells grow out to the right and left of the apices of the central cells in a direction of growth perpendicular to that of the central cells. The central cells are smooth, the lateral cells densely covered with blunt spines. Measurements of the conidium when seen in top view 14 (12—15) $\mu$ diameter, in side view 14 (12—15) × 7.5—10 $\mu$.

On decaying plant material in water.

The generic name for this fungus is suggested by the similarity of the conidium to a chandelier. Because of the branched and more or less stellate structure of the conidium it is suggested that the genus *Candelabrum* should be placed in the group *Staurosporae* of the Fungi imperfecti.

Type material will be deposited at Kew Herbarium. Living and dried cultures are maintained at the Centraal Bureau voor Schimmelcultures, Baarn.

## Summary.

A new monotypic genus, *Candelabrum*, is described. The fungus *C. spinulosum* was isolated from decaying leaves in water. The conidia are produced just above the water level on little differentiated conidiophores. Each conidium consists of 4 central cells and 8 lateral cells which are covered with blunt spines. The resemblance of the conidium to a four-armed chandelier suggests the generic name.

## Zusammenfassung.

*Candelabrum* wird als neue Hyphomyceten Gattung der *Staurosporae* beschrieben. Die Typusart *C. spinulosum* wurde auf einem faulenden im Wasser liegenden Blatt gefunden. Die Konidien entstehen direkt über dem Wasser an wenig differenzierten Trägern. Eine einzelne Konidie besteht aus vier zentralen und acht lateralen Zellen, sodass sie in ihrer Form an einen Kandelaber erinnert. Die äusseren Sporenzellen sind mit feinen Höckerchen besetzt.

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# SEROLOGICAL INVESTIGATIONS ON THE PHASEOLUS VIRUSES 1 AND 2 [1]

by

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(Received June 30, 1951).

## INTRODUCTION.

The common mosaic of French bean is a virus disease that has been known for a long time. SMITH (10) classified the causal agent as Phaseolus Virus 1. The fact that this virus may be transmitted by seed to the progeny of a diseased plant is undoubtedly one of the factors responsible for the world-wide occurrence of the virus (9).

Although some authors (7, 8) claim that Phaseolus Virus 1 may infect not only *Phaseolus vulgaris* but also other *Phaseolus* species and even *Vicia faba*, other investigators (3, 13) consider that *Phaseolus vulgaris* is the only host plant. In inoculation experiments the present authors failed to infect *Vicia faba* with this virus.

The symptoms caused by the virus depend on the bean variety and on environmental conditions. Some varieties react mainly with mosaic and light to heavy curling of the leaves; others show more or less severe necrosis (called "steengrauw" in Dutch) and some develop "black root" (12).

The yellow bean mosaic, classified by SMITH (10) as Phaseolus Virus 2, is less common. It has been described from the U.S.A. (5); in Europe it has been found, so far as we know, only in Belgium (11) and the Netherlands (12). This virus differs in two points from Phaseolus Virus 1; it is not transmitted by the seed of an infected plant, and it has a wider hostrange. Thus the virus infects several

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[2] The authors feel greatly indebted towards Dr G. G. SAMUEL, Harpenden, who corrected the English of the text.

leguminous plants (5, 12), and in the U.S.A. it has been found that *Gladiolus* may carry it (6).

The symptoms of this virus also, are dependent on variety and on environmental conditions. Some bean varieties react with a more or less pronounced mosaic; others show top-necrosis, and sometimes symptoms are observed which are hardly distinguishable from the "steengrauw" caused by Phaseolus Virus 1.

Inactivation temperature, dilution end-point and longevity *in vitro* are the same for both viruses, *viz.*, about 58° C., $10^{-3}$ and between 24 and 48 hours respectively. Both viruses may be transmitted mechanically as well as by aphids. GROGAN and WALKER (4) have investigated in premunity studies the relationship between both viruses. Although the number of test plants was relatively low, GROGAN and WALKER concluded that bean plants infected with Phaseolus Virus 1 acquired premunity against Phaseolus Virus 2. Conversely, the last-mentioned virus protected bean plants, though to a lesser degree, against infection with Phaseolus Virus 1. From this more or less mutual exclusion effect it may be concluded that the viruses are related.

To obtain an exact picture of the relationship between two viruses an investigation of the serological behaviour of each is necessary, besides the premunity studies. The present writers therefore examined whether it was possible to obtain antisera against the Phaseolus Viruses 1 and 2, and further whether these viruses would react with the heterologous antisera.

In connection with this serological investigation it was considered desirable to study whether or not Phaseolus Virus 1 is present in clear-centrifuged sap of diseased French bean plants. This was done by an inoculation experiment, using *Phaseolus vulgaris* var. Beka as a test plant.

## MATERIALS AND METHODS.

Phaseolus Virus 1 was isolated from a field-grown French bean plant showing common bean mosaic. Sap inoculations on *Vicia faba*, which were without result, showed that this virus was not contaminated with Phaseolus Virus 2. The virus was multiplied in the French bean variety Beka. Phaseolus Virus 2 was represented by a strain isolated in 1949 from a French bean plant which showed topnecrosis. Unlike the strain described in a former paper (12), which caused only a very faint mottling in *Vicia faba* and *Pisum sativum*, this one was characterized by a distinct mosaic in these plants. The virus was maintained in *Phaseolus vulgaris* var. Beka and *Vicia faba* var. Driemaal Wit.

To obtain antisera against both viruses, rabbits were intravenously injected with sap from diseased plants, *viz.*, *Phaseolus* beans, var. Beka, infected with Phaseolus Virus 1 and broad beans, var. Driemaal Wit, infected with Phaseolus Virus 2 respectively. The plants were crushed in a mortar, the sap expressed by hand and filtered through filter paper. Afterwards the sap was dialyzed in cellophane for three or four hours in running tap water to free it from toxic substances which might be present in it. The dialyzed sap was injected without further treatment. The rabbits received four injections per week, with three days interval at the week-ends. Three rabbits were injected with Phaseolus Virus 1; two with Phaseolus Virus 2. Every animal received a total of about 45 ml during 12 injections, without disadvantageous consequences. One week after the last injection 50 ml of blood was taken from an ear vein of each animal. This was repeated during the next two days. The serum was separated from the blood in the ordinary way and was kept at —24° C.

Saturation of the sera was done with uncentrifuged sap from healthy Beka or Driemaal Wit plants. It was found that saturation took place best by using sap previously diluted in equal volume with saline. The Phaseolus Virus 1 antiserum proved to be saturated after mixing one volume of serum with 15 volumes of diluted sap; and Phaseolus Virus 2 antiserum after mixing one volume of serum with 24 volumes of diluted sap. The mixtures were kept at room temperature for 5 hours and then centrifuged. The clear supernatant liquids were used in the serological experiments, which were carried out according to the micromethod of VAN SLOGTEREN and cow orkers (2).

This method is carried out by mixing one drop of serum with one drop of sap on a cover slip. The cover slip is then mounted on a small glass ring that has been stuck on a microscope slide in the same way as a moist chamber is prepared. The serological reaction takes place in the hanging drop. After incubation at 37° C. the result of the reaction may be examined microscopically. In our experiments examination took place after 20 or 30 minutes.

Precipitation as well as agglutination tests were investigated.

For the first tests, the sap of diseased and healthy plants was centrifuged for 20 minutes at 12.000 r.p.m. The reactions were carried out in series, in which dilutions of saturated antiserum were combined with dilutions of sap. Normal serum, previously treated with sap from healthy plants (haricot and broad beans) in the same way as the antisera, served as check. Within 5 hours the mixtures antiserum-healthy sap showed a strong coagulation of plastids and other solid sap components, whereas the mixtures normal serum-healthy sap showed no coagulation. Saline, which was used as a diluent, served as second check.

The infection experiments were carried out on Beka plants that had just unfolded their first leaves. Inoculations were performed on one day by the same person. Carborundum, 500 mesh, was used as an abrasive.

## RESULTS.

### § 1. Serological reactions with Phaseolus Virus 1 antiserum.

The results obtained with the sera of the three rabbits injected with Phaseolus Virus 1 did not differ very much. Several dilutions of the saturated antiserum were made, running from undiluted to the dilution 1 : 64. The same series was made from normal serum, treated with the sap of healthy plants. The dialyzed and centrifuged sap of Beka plants infected with common bean mosaic and of healthy Beka plants was combined in the undiluted form and in the dilutions 1 : 2 and 1 : 4, with this series. This experiment was carried out late in the summer of 1950. The serum sap mixtures were kept at 37° C. for 20 to 30 minutes and then examined microscopically. It was found that all dilutions of the sap of healthy plants with the antiserum and normal serum series had remained clear; white, although rather faint, precipitates had formed in the mixture of the undiluted centrifuged sap of mosaic diseased bean plants with the antiserum range. A very faint reaction was obtained with one of the three saturated antisera in a dilution of 1 : 32, corresponding with a serological titre of $1/16 \times 1/32 = 1 : 512$. Thus calculated, each of the other two sera had a titre of 1 : 256.

It is remarkable that the diluted sap of the diseased plants showed no visible reaction. Normal serum mixed with the sap of diseased plants did not give a positive reaction.

In an experiment using undialyzed sap, it was found that spontaneous precipitates occurred in the mixtures serum-"healthy" sap. Conditions for obtaining good results in the precipitin reaction are thus to dialyze the sap previously and secondly to avoid dilution of the sap.

Furthermore, it appeared that saturation of the antiserum can best be done at room temperature. Keeping the mixture antiserum-sap of healthy plants at 37° C. for 3 hours may result in the destruction of the virus antibodies. Antiserum treated in this way did not show any positive reaction with the sap of diseased plants. Finally, it was found that the saturated antiserum stored at 1° C. may be kept only for two to three days. Serological activity proved to be lost after this period.

The results obtained lead to the conclusion that the quantity of virus in clear-centrifuged sap of mosaic diseased beans is rather small, as even the 1 : 2 diluted sap does not show a positive reaction. On the other hand the titre of the sera is rather high, or in other words the sera contain a considerable quantity of antibodies against the virus. Obviously much of the virus is sedimented during centrifugation. This conclusion was tested and confirmed in an inoculation experiment to be described in § 4.

The same antisera were also tested in the agglutination reaction. The dialyzed sap of healthy and diseased Beka plants was mixed undiluted and in the dilutions 1 : 2 and 1 : 4 with the saturated antiserum, which was diluted with different quantities of saline. Normal serum, previously treated with sap of healthy plants, and saline, served as checks. It was found that the sap of the diseased bean plants contained much starch, even when the plants were picked early in the morning. It is possible that the normal transport of sugars may be disturbed as a consequence of the disease. The starch grains showed a high tendency to agglutinate, which made the result of the serological reaction hard to judge. A centrifugation of the sap at low speed for one or two minutes before mixing with antiserum removed the bulk of the starch, without precipitating the other solid sap components. Agglutination of plastids was sometimes difficult to observe. It is noteworthy that it occurred distinctly in the sap of diseased plants grown in the glasshouse during August and September 1950 and April 1951, but that no positive agglutination tests were obtained in the sap of diseased beans grown during the winter months.

### § 2. Serological reactions with Phaseolus Virus 2 antiserum.

For the precipitin test, dialyzed and centrifuged sap, in this case of diseased broad bean plants, was used. The experiment was carried out in the autumn of 1950. The reactions with the undiluted sap of diseased *Vicia* plants were positive, even at high dilutions of the antiserum (both sera had a dilution end point of 1 : 32, which corresponds with a titre of 1/25 × 1/32 = 1 : 800. Furthermore, only the sap of diseased plants in the dilution 1 : 1 with saline reacted positively with the undiluted saturated antiserum.

The agglutination reaction of Phaseolus Virus 2 antiserum with sap of *Vicia faba* infected with this virus was very distinct (see Fig. 1). No disturbing starch was present in the sap. The saturated antiserum, diluted 1 : 16 with saline, still gave a positive agglutination reaction with undiluted sap of diseased broad bean. Thus the titre of this antiserum, defined in the agglutinin test, lays between 1 : 400 and 1 : 800. Normal serum and saline did not produce any agglutination of the plastids.

Contrary to the experience obtained in the study of Phaseolus Virus 1 anti-sera, all diseased *Vicia* plants tested showed a distinct positive reaction in autumn and winter as well as in spring.

The agglutination reaction of the saturated Phaseolus Virus 2 antiserum was further tested with the sap of Beka plants infected with this virus. This sap was freed from superfluous starch grains by a short low-speed centrifugation. It is noteworthy that the result of this reaction was also found to depend on the season in which the French beans were grown. In the autumn of 1950 and in the spring of 1951 positive reactions were obtained with the sap of Beka beans infected with Phaseolus Virus 2, whereas all tests with the sap of winter-grown infected bean plants remained negative.

### § 3. Study of the two viruses with the heterologous antisera.

Owing to the fact that the sap of Beka plants infected with one of the two viruses does not react visually with its homologous antiserum during winter time, one might suppose that in the case of a possible serological relationship between the Phaseolus Viruses 1 and 2 there might be a chance that the saturated Phaseolus Virus 1 antiserum would not give a reaction with the sap of Beka plants infected with Virus 2, and also that the saturated antiserum against

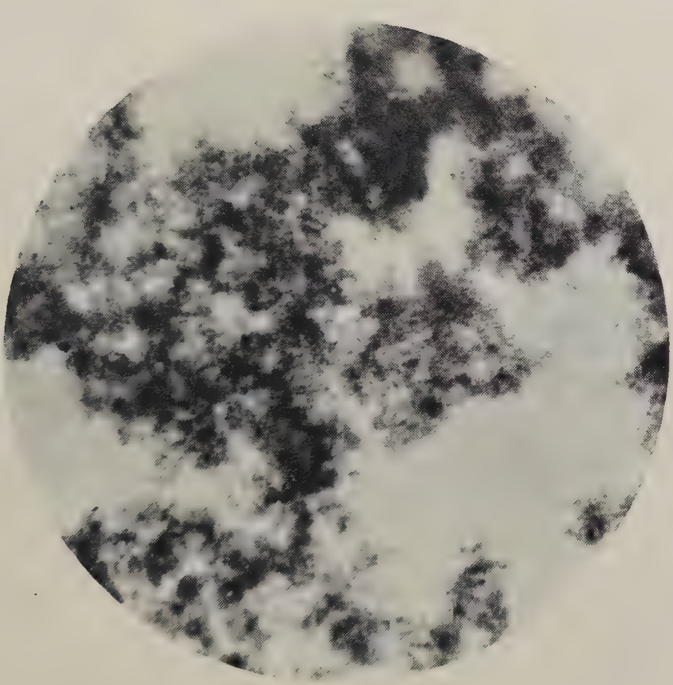

Fig. 1*a*. Sap of *Vicia faba* var. Driemaal Wit, infected wit Phaseolus virus 2, mixed with Phaseolus Virus 2 antiserum, saturated with sap of healthy *Vicia faba*.

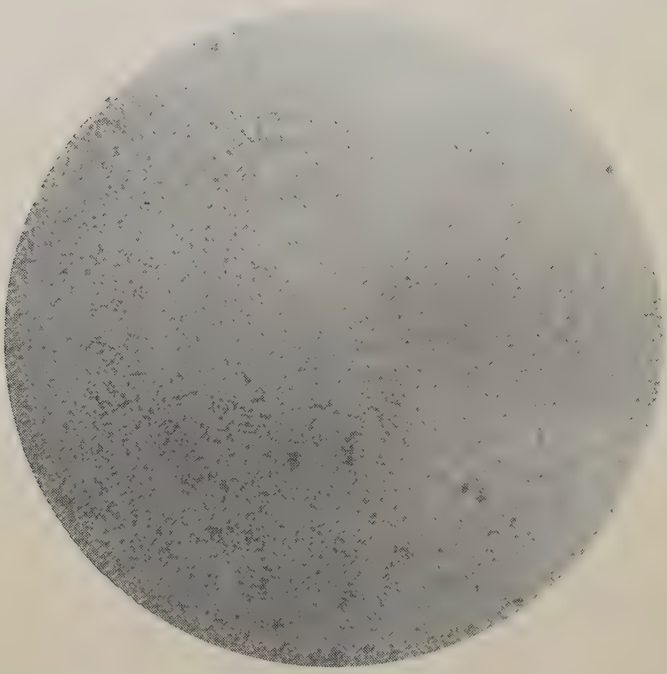

Fig. 1*b*. Sap of healthy *Vicia faba* var. Driemaal Wit, mixed with Phaseolus Virus 2 antiserum, saturated with sap of healthy *Vicia faba*.

Virus 2 would not react with the sap of Beka plants infected with Virus 1. This proved to be true during the winter months. However, faint positive agglutination reactions were obtained during April 1951. The antiserum against Phaseolus Virus 1 always caused a distinct positive agglutination of plastids in the sap of *Vicia faba* infected with Phaseolus Virus 2. This shows that there exists a serological relationship between the two viruses.

## § 4. Inoculation experiment.

In order to verify the indication that very little virus is present in the centrifuged sap of Beka plants infected with Phaseolus Virus 1 (§ 1), an inoculation experiment was carried out during the autumn of 1950. The infectivity was estimated of: (1) sap of diseased Beka plants; (2) sap of the same plants clear-centrifuged for 20 minutes at a speed of 12.000 r.p.m.; (3) the sediment, obtained after centrifugation, resuspended in distilled water to the original volume of sap. As bean leaves do not react with local lesions after inoculation with Phaseolus Virus 1, the virus concentrations in these three objects were estimated in dilutions, *viz.*, undiluted, $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$. The undiluted sap and the dilutions were each inoculated into the primary leaves of about 45 bean plants. The results of the experiment are summarized in Table I.

Table I. 1)

| | dilutions | | | | |
|---|---|---|---|---|---|
| | un-diluted | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| Untreated sap | 32/45 | 17/48 | 12/45 | 2/45 | 0/44 |
| Clear-centrifuged sap | 0/43 | 0/46 | 0/41 | 0/45 | 0/39 |
| Sediment, resuspended in water | 41/44 | 25/44 | 6/45 | 0/44 | 0/43 |

1) The denominator shows the number of inoculated French bean plants; the numerator the number of plants showing symptoms.

It was concluded from this experiment that the clear-centrifuged sap was no longer infectious, the infectivity of the uncentrifuged sap having gone into the sediment. This result agrees with the results of the serological precipitin reactions, described in § 1.

## DISCUSSION.

It has been shown that it is possible to obtain antisera against the Phaseolus Viruses 1 and 2 by injecting rabbits intravenously with dialyzed sap of virus diseased plants. Phaseolus Virus 2 can be demonstrated easily in the sap of *Vicia faba* var. Driemaal Wit, infected with this virus, using the agglutination test. It is more difficult to prove that antibodies against Phaseolus Virus 1 are present in its antiserum, as the reaction is sometimes inhibited by a factor in the sap of *Phaseolus vulgaris* var. Beka. With respect to this it has been shown by BAWDEN and PIRIE (1), that by milling the fibre of tomato plants in a triple roller mill a substance may be liberated that inhibits precipitation of the tomato bushy stunt virus with its antiserum. Furthermore, BAWDEN and PIRIE (1) showed that an analogous inhibitor is present in *Phaseolus vulgaris* var. Canadian Wonder. In our case a factor is concerned which inhibits agglutination. This inhibiting effect is most strongly developed when the sap is used from plants which have been grown during the winter months. It remains uncertain in which constituent of the sap this inhibiting factor is localized.

The results of the agglutination tests between Phaseolus Viruses 1 and 2 and their heterologous antisera indicate that the two viruses are serologically related. The reactions gave no information about the degree of relationship, as the study of this was hampered by the presence in the sap of Beka plants of the abovementioned factor which inhibits the agglutination reaction. It may also be concluded from the work of GROGAN and WALKER (4), who found the two viruses to have a certain mutual premunizing effect in French beans, that these viruses are related.

Furthermore, it was found in the autumn of 1950, with the help of precipitin tests, that very small quantities of virus are present in the clear-centrifuged sap of *Phaseolus vulgaris* var. Beka, infected with Phaseolus Virus 1. An inoculation experiment with clear-centrifuged Beka sap gave good evidence, that this is not due to the action of a precipitation inhibiting agent. Although the inoculations remained negative, it was found that the sediment, obtained after centrifugation of the sap, showed the same infectivity as the uncentrifuged sap.

## Summary.

1. Antisera against the Phaseolus Viruses 1 and 2 were obtained by injecting rabbits intravenously with uncentrifuged, dialyzed sap from *Phaseolus vulgaris* var. Beka, infected with Phaseolus Virus 1 and from *Vicia faba* var. Driemaal Wit, infected with Phaseolus Virus 2.

2. These antisera gave faint precipitin reactions with the clear-centrifuged sap of plants infected with the respective viruses.

3. The antiserum against Phaseolus Virus 1 gave faint agglutinations with the sap of *Phaseolus vulgaris* var. Beka, infected with Phaseolus Virus 1 during autumn and spring. During the winter months all reactions remained negative.

4. Distinct agglutinations in the sap of *Vicia faba* var. Driemaal Wit, infected with Phaseolus Virus 2 were obtained, not only in autumn and spring, but also in winter time, after mixing the sap with the antiserum against Phaseolus Virus 2. The sap of *Phaseolus vulgaris* var. Beka, infected with the same virus, behaved in the same way as sap infected with Phaseolus Virus 1. All reactions of the infected saps with normal serum remained negative.

5. Distinct agglutination of the solid components in the sap of the broad bean variety Driemaal Wit, infected with Phaseolus Virus 2, occurred after mixing with antiserum against Phaseolus Virus 1. The antiserum against Phaseolus Virus 2 gave positive reactions with the sap of French bean plants, var. Beka, infected with Phaseolus Virus 1, only when the plants were grown in autumn or spring. During the winter the reactions remained negative. From these results it is concluded that the Phaseolus Viruses 1 and 2 are serologically related.

6. The sap of *Phaseolus vulgaris* var. Beka, infected with one of the viruses mentioned, apparently contains a factor which inhibits agglutination of the solid sap components after mixing with the saturated homologous or heterologous antiserum. The effect of this factor is very marked in the sap of plants grown during the winter months. The effect is less during spring and autumn, since rather faint reactions may be given at those times of the year. A similar factor is not present to any marked degree in the sap of *Vicia faba* var. Driemaal Wit.

7. Phaseolus Virus 1 proved to be associated to a large extent with the solid sap components.

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# CHITIN IN THE CELL WALL OF YEASTS

by

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## INTRODUCTION.

In 1937 Prof. A. J. KLUYVER encouraged one of us (HOETTE) to investigate the correctness of SCHMIDT's (21) hypothesis that chitin occurs in all filamentous yeasts and is absent in non-filamentous ones. During the investigation which followed, in fact chitin was demonstrated in many, but not in all of the 29 species tested. However, it was not a characteristic feature of filamentous or non-filamentous yeasts, nor of ascogenous or non-ascogenous ones. It seemed likely that chitin occurred in all yeasts, but in varying and sometimes very small amounts, the main cell wall constituents being different. The war was responsible that these results remained unpublished.

When recently the study of the structure and the composition of the yeast cell wall was taken up again in our laboratories (10) the pre-war results were checked and extended by the senior author (ROELOFSEN). The combined results, as far as the chemical demonstration of chitin is concerned, will be described in this paper.

## § 1. SURVEY OF LITERATURE.

This is confined to the more important publications on the composition of the cell wall after extraction with alkali. SCHLOSSBERGER (20) extracted brewer's yeast at room temperature with dilute alkali for several days and obtained a residue containing only 0.5% N, with C, H and O contents similar to starch and producing a fermentable sugar after prolonged boiling with dilute sulfuric acid.

SALKOWSKI (19) extracted the so-called yeast gum from baker's yeast by boiling with 3% KOH for half an hour. On heating the residue in water at 120—130°C. for 20 hrs about half of it dissolved and yielded glucose on hydrolysis. The insoluble half was reported to yield glucose and mannose.

MEIGEN and SPRENG (13) confirmed and extended these results, using brewer's yeast. Again a glucose-polymer was extracted from the alkali treated yeast either by SALKOWSKI's method, or by prolonged boiling in 15% alkali or 3% sulfuric acid. On hydrolysis of the final, insoluble residue 3 mannose to 4 glucose units were obtained (demonstrated respectively as phenylhydrazon and phenylosazon). Like SALKOWSKI and SCHLOSSBERGER the authors found a very low nitrogen content in the final residue and therefore considered chitin to be absent.

VAN WISSELINGH (23) had already reported the absence of chitin in baker's yeast. He cleaned the yeast by heating in glycerol at 300°C. and observed that practically nothing was left. Chitin will stand this treatment. If the yeast was heated at 160°C. in 60% KOH and GILSON's reaction on chitosan (6), *viz.*, a violet colour with IKI in dilute sulfuric acid, was applied, only a brown colour was obtained. VON WETTSTEIN (25) and many others confirmed this.

SCHUMACHER (22) extracted ground baker's yeast with cold dilute alkali, water and boiling alcohol and then qualitatively demonstrated a polysaccharide and a protein in the cell wall residue, which, however, was obviously cleaned insufficiently. Taking into account MEISENHEIMER's earlier demonstration of glucosamin in (likewise inadequately cleaned) yeast cell wall material, he rashly postulated the presence of a glucosamin containing phospho-gluco-protein.

ZECHMEISTER and TÓTH (27) again confirmed the low nitrogen content in baker's yeast successively treated with boiling alkali, HCl, alcohol and ether and therefore concluded to absence of chitin. Neither mannose, nor a ketose, but only glucose could be found after hydrolysis of the residue. Finding 2, 4, 6 trimethylglucose as the main cleavage product of the methylated residue, 1—3 bonds between the glucose radicals were tentatively postulated.

SCHMIDT (21) was the first to claim the presence of chitin in certain yeasts, *viz.* in *Endomycopsis capsularis* Schiönning, *E. fibuliger* Lindner and *Eremascus fertilis*, all belonging to the filamentous *Endomycetaceae*. In the residue after heating at 100°C. for several hours with 5—20% alkali and with 1% HCl and after treatment with $KMnO_4$, oxalic acid, alcohol and ether, more than 6% N was found. Moreover chitin was dissolved in cold conc. HCl and precipitated in water. Glucosamin was isolated as the tetra-benzoyl derivative. In the non-filamentous *Saccharomyces cerevisiae* var. *marchalianus* the presence of chitin was considered questionable, although the cleaned cell wall material was reported to contain 4.3% N! SCHMIDT suggested that contrary to filamentous yeasts, the non-filamentous ones do not contain chitin.

Following this publication, the investigations of one of us (H.) as referred to in the Introduction, were performed.

Later, NABEL (15) applying VAN WISSELINGH's method, confirmed the presence of chitin in the three filamentous yeasts analysed by SCHMIDT, as also in two *Endomyces* species, in *Pichia membranaefaciens* and in *Saccharomycodes Ludwigii,* but not in five other yeasts, including *Saccharomyces cerevisiae.*

HASSID *et al.* (8) confirmed the conclusion of ZECHMEISTER and TÓTH, that the residue of baker's yeast after boiling with dilute alkali and HCl was a polymer of glucose with 1—3 $\beta$ bonds. No endgroup was detected and no signs of branching. Again, no mannose-phenylhydrazon could be obtained in striking contrast to the results of SALKOWSKI and of MEIGEN and SPRENG. Since the nitrogen content of the residue was only 0.56% and practically nil after acetylation, it was considered as an impurity.

BARRY and DILLON (1) obtained further evidence of the 1—3 $\beta$ linkage of the glucose polymer in alkali-treated baker's yeast. They found one endgroup on 28 radicals as against one on sixteen in laminarin, a soluble 1—3 $\beta$ glucan.

BELL and NORTHCOTE (2) digested baker's yeast with 6% alkali at 60°C. and with 3% at 80°C., then treated the residue with 0.5 N acetic acid at 75°C. After hydrolysis only glucose could be detected on a paper chromatogram. With the method used mannose, if present, presumably should have been detected. The cleavage products of the methylated glucan were analysed after chromatographic separation and led to the conclusion that there was one endgroup in every nine radicals, one of the nine bonds being of the 1—2, the others of the 1—3 type. Tests on pentoses and uronic acids were negative as also HASSID *et al.* had stated. The nitrogen content of the cell wall residue was 0.42% and practically nil after methylation.

FREY (5) confirmed SCHMIDT's results as regards occurrence of chitin in filamentous *Endomycetaceae* and absence in other yeasts. Applying the X-ray identification method on yeast cell walls, cleaned by repeated boiling with 10% KOH and water, he could detect chitin interferences in the residues of three species, in two of which chitin had also been demonstrated by Schmidt and by Nabel.

With 13 yeasts belonging to other groups, including *Saccharomyces cerevisiae,* no chitin lines were seen, but a pattern belonging to an inorganic substance, probably $BaSO_4$. In addition a 7.7Å line was usually present and sometimes additional unidentified interferences. In view of the possible occurrence of amorphous chitin in these cases, the residue was extracted with cold 24% HCl and the extract was diluted with water. If chitin had been present, a crystalline chitin precipitate should have been formed. However, no such precipitate was obtained.

The Van Wisselingh chitosan-iodine test, applied on yeast residues producing no chitin interferences, was usually negative, but sometimes positive. It could even be positive with residues freed from chitin by extraction with concentrated HCl. Therefore the test was considered as wholly unreliable.

The relevant facts, claimed in literature, may be summarized as follows.

A characteristic glucan, different from cellulose and starch, is the main constituent of the cell wall residue of baker's yeast after extraction with hot alkali and acid. In the hydrolysed residue ketoses, pentoses and uronic acids are absent. Two authors claim to have found mannose in addition to the glucose, but others could only detect glucose.

Since the nitrogen content of the residue of both baker's and brewer's yeast is low, absence of chitin is generally accepted. This is also claimed for pure cultures of *Saccharomyces cerevisiae* and other non-filamentous yeasts on the grounds of absence of chitin X-ray interferences and of negative results with the chitosan-iodine test.

However, in several *Endomyces* and *Endomycopsis* species the occurrence of chitin has been indicated by macrochemical analysis as well as by positive chitosan-iodine tests and definitely demonstrated by its X-ray interferences.

## § 2. EXPERIMENTAL.

Except when baker's or brewer's yeast was used, the material was collected from pure cultures of yeast species obtained from the Central Bureau of Yeast Cultures at Delft. They were always cultivated on malt extract agar at 25°C.

### *a.* Chitosan-iodine tests.

The de-acetylation of possible chitin was carried out according to VAN WISSELINGH. A small quantity of yeast was heated in 60% KOH (w/v basis) in fused small glass tubes at 160°C. for 15 min. No oil-bath was used as VAN WISSELINGH recommends, but the tubes were enveloped with copper wire gauze and kept in a kind of copper wire basket in a wide glass tube above a gas burner, a thermometer indicating the temperatures reached. Then the tube contents were mixed with 96% alcohol in a centrifuge tube and consecutively washed with 40% alcohol and water.

On a slide the residue was treated with dilute IKI and 1% sulfuric acid. If the alkali treatment was applied without pretreatment of the yeasts, all colours between violet and brown occurred depending on the amount of glycogen left. Pretreatment with glycerol at 300°C. or at lower temperatures was unsuccessful, since either brown oily droplets, or a charred sticky mass was obtained. Apparently no such difficulties were encountered by NABEL and FREY. Starving and autolysis of the yeasts was helpful, but oxidation with 1% $KMnO_4$ at room temperature was better and easier. After centrifugation, the manganese containing residue was treated with hot 1% HCl in saturated oxalic acid and washed with water. Oxidation after alkali treatment is unsuitable since chitosan is attacked, a fact already mentioned by LÖWY (12).

A violet, reddish violet or brownish violet colour was considered as a positive, a brown colour as a negative indication for chitosan. As appears from table 1, the test was positive with 20 yeast species; with three the result was doubtful and six were negative. Positive and negative results were found both in ascogenous and non-ascogenous species as well as in so-called filamentous and in non-filamentous yeasts. However, like several authors, we are of opinion that the evidence of the chitosan-iodine test is unsatisfactory since the appreciation of the slight colour differences is too subjective.

### *b.* Chitosan sulphate tests.

1. Introductory remarks. It is remarkable, that apparently no published attempts have been made to obtain crystals of chitosan salts from yeasts, although the method, extensively used for detection of chitin in fungi and insect cuticles, was already described in the 1923 edition of Molisch' well-known handbook "Mikrochemie der Pflanzen".

Crystalline chitosan salts were already known to Gilson (6), but their characteristic shape was first mentioned by Hoppe Seyler (9) and described in more detail by Von Fürth and Russo (24), while Löwy enlarged our chemical knowledge of chitosan sulphate. To Brunswik (3) we are greatly indebted for the description of a microchemical method for obtaining them from chitosan prepared according to Van Wisselingh. Brunswik brought fungal or insect chitosan in 10% $H_2SO_4$ on a slide, applied a coverglass and dissolved the chitosan by heating with a microburner to the boiling point. Then the slide was cooled very slowly on a sandbath or in a thermostat of initially 80°C. Flat circular birefringent sphaerocrystals of 8—25 $\mu$ diameter, showing the Brewster cross, were obtained. Without coverglass more often tetragonal sphaerocrystals, dumbbell-shaped on side view were encountered as well as sickle-shaped smaller crystalls, both of these being already described by Von Fürth and Russo. Rapid cooling produced a mass of minute crystalline particles. The crystals already recognizable by their typical shape, were also characterized by staining brown with iodine dilute acid, and by their vivid coloration with dilute picric acid, acid fuchsin and other aniline dyes.

Campbell (4) after extensive experimenting strongly recommended a modified Van Wisselingh-Brunswik test for detecting chitin in insect cuticles. He introduced two modifications: 1. heating at 160°C. in open tubes with KOH solution, saturated at room temperature, 2. crystallization of chitosan sulphate from a solution in cold concentrated sulfuric acid by allowing this solution to attract water vapour, instead of slowly cooling down a hot solution in dilute acid.

According to Glenn Richards (7) he and others have encountered difficulties in attempts to prepare consistently the characteristic sphaerocrystals from insect cuticles which undoubtedly did contain chitin. Moreover, he stated, that "the sphaerites have such indefinite characteristics, that by themselves they do not seem highly reliable" (as a chitin test). FREY mentioned this view in motivating his rejection of the method, but he did not try it himself. However, as will be shown later on, the test is highly reliable provided only the occurrence of characteristic birefringent sphaerocrystals is taken as evidence.

Crystalline chitosan sulphate is probably not homogeneous, since the rigourous alkali treatment of chitin leads to a mixture of polyglucosamines of on the average about 15 radicals only (14). This depolymerisation is necessary for obtaining crystallizing salts (18).

2. Results. The tests done in 1937 by one of us (H) were performed with chitosan, obtained from precleaned yeast, as was also used for the chitosan-iodine test. The crystals were obtained by following BRUNSWIK's prescription. Of 29 yeasts tested and listed in table 1, 15 were positive, 2 doubtful, 12 negative. Of the latter, however, 3 were positive with the chitosan-iodine test, but, admittedly, this test is not fully reliable by itself. Since apparently closely related species produce different results with the chitosan sulphate test, it was assumed that chitin occurred in all yeasts, but in varying and sometimes not-demonstrable amounts.

This suggestion was essentially confirmed by the chitosan sulphate tests performed by the senior author recently with some of the yeasts studied earlier, including all those, having given negative or doubtful results previously. As appears from table 1, only one yeast species is persistently negative.

Two reasons readily explain this divergence of results. Firstly much more yeast was used in the tests and the residue was treated with as little sulfuric acid as was sufficient to prepare a few mounts, while in the earlier experiments much more acid was used.

Secondly, if initially negative results were obtained, the tests were repeated several times using material from old as well as from young cultures. Cultural conditions seem to influence the chitin content. For instance the crystals are easier to obtain from baker's yeast than from a pure culture isolated from it and cultivated on malt extract agar. Moreover, old cultures seem to contain more chitin than young ones.

The failure of previous authors to demonstrate chitin in most yeast species, including baker's yeast, and the positive results re-

TABLE I.

Microchemical tests on chitin in yeasts.

| Yeast species | Early tests (Hoette) | | Recent tests (Roelofsen) |
|---|---|---|---|
| | chitosan-iodine | chitosan sulphate | chitosan sulphate |
| Non-ascogenous group. | | | |
| *Candida albicans* (Robin) Berkhout | + | ? | + |
| *Candida tropicalis* (Cast.) Berkhout | + | + | + |
| *Candida krusei* (Cast.) Berkhout | + | + | + |
| *Candida parapsilosis* (Ashf.) Langeron et Talice | + | + | |
| *Candida scottii* Diddens et Lodder | + | + | |
| *Candida pelliculosa* Red. | + | — | + |
| *Candida lipolytica* (Harrison) Diddens et Lodder | + | + | + |
| *Trichosporon pullulans* (Lindner) Diddens et Lodder | + | + | |
| *Schizoblastosporion starkeyi-henricii* Ciferri | + | — | + |
| *Trigonopsis variabilis* Schachner | + | — | + |
| *Torulopsis colliculosa* (Hartmann) Saccardo | + | + | + |
| *Torulopsis holmii* (Jörg.) Lodder | ? | — | + |
| *Torulopsis laurentii* (Kuff.) Lodder | + | ? | + |
| *Rhodotorula mucilaginosa* (Jörg.) Harrison | — | — | + |
| Ascogenous group. | | | |
| *Saccharomyces intermedius* Hansen | — | — | + |
| *Saccharomyces chevalieri* Guilliermond | + | + | |
| *Saccharomyces cerevisiae* (Hansen) Dekker | + | + | + |
| Baker's yeast from ,,Kon. Ned. Gist en Spir.fabr.", Delft | | | + |
| Pure culture from this baker's yeast | | | + |
| Brewer's yeast from Z.H.B. brewery, The Hague | | | + |
| *Saccharomyces cerevisiae* var. *ellipsoideus* (Hansen) Dekker | — | — | + |
| *Saccharomyces cerevisiae* var. *marchalianus* (Kuferath) Dekker | — | + | |
| *Saccharomyces carlsbergensis* Hansen | + | + | |
| *Zygosaccharomyces japonicus* Saito | + | + | |
| *Pichia belgica* (Lindner) Dekker | ? | — | + |
| *Hansenula anomala* (Hansen) Sydow | — | — | + |
| *Endomycopsis capsularis* (Schiönning) Dekker | + | + | |
| *Schizosaccharomyces octosporus* Beijerinck | — | — | — |
| *Zygosaccharomyces lactis* | ? | — | + |
| *Zygosaccharomyces bisporus* Naganishi | + | — | + |
| *Debaryomyces membranaefaciens* Naganishi | + | + | |
| *Debaryomyces tyrocola* Konokotina | + | + | + |

ported with certain other species, is readily explained by the apparently greatly varying amount of chitin present in different species. As is to be deduced from the difference in easiness of obtaining the chitosan sulphate crystals, most *Candida* species for instance contain more chitin than *Saccharomyces* species.

Admittedly, our negative results with *Schizosaccharomyces octosporus* might be merely circumstantial, but a true genotypical unability of producing chitin seems more probable. The tests with this yeast were repeated no less than eight times, using cultures of different ages and grown on malt agar at temperatures between 18 and 30°C. Twice, considerable quantities of well cleaned cell wall material were used. There are indications that also in other respects the cell wall composition differs from that of other yeasts.

Up till now we tacitly assumed that if chitin was demonstrated, this had been part of the cell wall. Although this assumption needs little comment, we have in fact demonstrated chitin in the cell wall residue obtained by boiling baker's yeast and other yeasts with dilute alkali and acid according to ZECHMEISTER and TÓTH. This residue contained 0.9% N, as against 0.4—0.6% in a similar residue obtained by previous investigators. These figures account for at most 6—12% chitin. Probably the amount is even less, for cells of baker's yeast cleaned in this way, still enclose one or two highly refractive globules which might contain nitrogen.

## § 3. DESCRIPTION OF RECOMMENDED METHOD FOR CHITIN DETECTION AND DOCUMENTATION OF ITS RELIABILITY.

### *a.* Obtaining chitosan sulphate crystals.

Several simplifications and modifications of the original VAN WISSELINGH-BRUNSWIK method (see § 2*b*, 1) of obtaining chitosan sulphate crystals were introduced in the course of our investigation.

Since the hot-glycerol precleaning method was unsuitable, we first used the same $KMnO_4$-oxidation-method, reported to be favourable for the chitosan-iodine tests (§ 2*a*). Later we observed that crystals were also obtained if the yeasts were subjected to the concentrated alkali treatment without any precleaning. However, previous removal of the bulk of the cell contents is preferable, since this reduces foaming in the hot concentrated alkali and decreases the non-chitosan fraction in the alcohol precipitate. Thus, more yeast may be used in a test, which enhances the sensivity of the method. The cell contents were most easily removed by extraction with hot dilute alkali.

Because fused glass tubes sometimes explode in the hot oil bath during the de-acetylation process, the senior author successfully used tin-plated steel tubes of 4 ml capacity, tightly closed with a screw and an annealed red copper gasket. However, the simpler method of CAMPBELL (4), *i.e.* heating in glass tubes provided with a BUNSEN valve and using a KOH solution saturated at room temperature, is to be preferred.

We tried to omit the use of alcohol for precipitation of the chitosan, but much chitosan seemed to be lost hereby. Furthermore the crystallization method introduced by CAMPBELL proved to be less suitable for our purpose than BRUNSWIK's method.

Thus, the following procedure is recommended. Two tubes or one Petri dish with malt extract agar are inoculated and incubated at 25°C. for at least a week. The crop is collected with water and the suspension is brought into a Pyrex tube of 8 ml capacity. After adding a few KOH granules, the tube is heated in a water bath for one hour. Subsequently this tube is centrifuged and the supernatant liquid is discarded. A few KOH granules, in volume approximately equal to the precipitated yeast residue, are added in addition to 3 ml KOH solution, saturated at room temperature (about 62% w/w basis). After closing the tube with a rubber stopper provided with a BUNSEN valve as described by CAMPBELL, the lower part is heated in glycerol or oil at 160°C. during 15 minutes. After cooling, the suspension is mixed in a larger centrifuge tube with at least 30 ml 96% alcohol and centrifuged. After a similar washing with 40% alcohol and several washings with water, the last time using a pointed centrifuge tube, the residue is carefully sucked in a glass tube with about 1 mm bore. A quantity of about 15 $mm^3$ is brought on each of three slides. Then a drop of 15% sulfuric acid is mixed with the residue and a coverglass is applied. After having warmed a sand bath at 80°C. the mounts are slowly heated with a microburner to an initial boil, immediately put on the sand and left to cool.

During this procedure the quantity of yeast material considerably decreases, because it partly dissolves in the alkali and during the subsequent washing with water. If there is too little left to apply the aforementioned procedure for preparing the mounts, one drop of sulfuric acid is brought in the pointed centrifuge tube and mixed with the chitosan containing residue. This suspension is sufficient for one or two mounts.

### *b.* Identification of the chitosan sulphate crystals.

Since many kinds of crystals were encountered in the mounts, a fact, which probably discouraged GLENN RICHARDS (7), and since the chitosan sulphate crystals were often scarce and minute, it was found to be absolutely necessary to use a polarisation microscope for searching the preparation and for studying the crystals with high magnification. A strong light source and a sensitive method for determining the character of the double refraction (11) was needed.

The presence of chitosan sulphate crystals was most easily detected by sucking 1 % aequous acid fuchsin or 1 % picric acid through the mount and searching with low magnification between crossed nicols for red, respectively yellow "stars". This, moreover, precluded confusion with other crystals, which are not stained. Pieces of dirt, however, are often coloured too. Therefore the coloured crystals should be examined in detail using high magnification.

The characteristic shape of the crystals should first be observed in a mount containing chitosan sulphate from lobster- or fungus-chitin. Then the similar, but usually smaller crystals obtained from yeast are easily recognised. Often there are many crystals of 1—3 $\mu$, either circular or rodlet-shaped. Usually the crystals are 3—10 $\mu$. When lying flat these are circular, when upright oval or with a small dent in the middle, like an 8, or like a dumbbell without handle (fig. 1*b*).

The conspicuously dented tetragonal sphaerocrystals, depicted in fig. 2, were seldom encountered by the senior author, but in the earlier experiments these were often found. Such differences in crystal shape have already been mentioned by VON FÜRTH and RUSSO and by BRUNSWIK. They might be due to varying crystallisation conditions as well as to different degrees of de-acetylation and depolymerisation.

In circular as well as tetragonal sphaerocrystals the BREWSTER cross — distorted if the crystal is not lying flat — is conspicuous (fig. 1*a*). In our opinion it is important to ascertain that they are negatively birefringent, the major refractive index being oriented transverse if the disks are standing upright and tangential if lying flat. This is illustrated by fig. 1*b*, which represents the same crystals as fig. 1*a*, likewise between crossed nicols, but with two quadrants compensated with a Leitz elliptic compensator. The retardation of a circular crystal of 5 $\mu$ diameter and 2.5 $\mu$ thickness standing upright, was about 48 m$\mu$.

In large sphaerocrystals a radial structure could be observed, indicating that they are aggregates of elongated crystallites oriented radially as is depicted in fig. 3. Since we may assume that the molecular chains in the crystallites will be oriented lengthwise, *i.e.* radially, these molecules must be negatively birefringent with respect to their axis as also chitin molecules are. This must be due to the transversely oriented amino groups.

### *c.* Reliability of the test.

Since GLENN RICHARDS and FREY doubted the reliability of the chitosan sulphate test, we wish to remark the following. To be sure, birefringent sphaerocrystals, staining readily with dyes, need not be chitosan sulphate crystals. YUNDT (26), for instance, described and depicted hexagonal xylan crystals also dumbbell-shaped on side view and stated that mannan crystals are similar. Sphaerocrystals from partly hydrolysed konjak-mannan have been described by OTUKI (16). GILSON's well-known cellulose crystals are also spherical and readily stain with iodine, congo red, etc. Inulin, amylose and native starch granules are similar in this respect. However, in most of these cases, the sphaerocrystals are known to be positively birefringent, as is to be expected with carbohydrates.

Furthermore, cellulose and chitin are the only cell constituents known to withstand a treatment with 60 % alkali at 160° C., but unlike chitosan, cellulose will not produce crystallizing salts with dilute sulfuric acid. It is very unlikely that any other native cell constituent will exhibit all of the following properties:

1. withstand boiling in dilute alkali and acid and heating in 60% alkali at 160° C.;
2. subsequently dissolve in hot dilute sulfuric acid and crystallize on cooling;
3. produce negatively birefringent sphaerocrystals of characteristic shape, that will take up iodine and will stain fast with picric acid, acid fuchsin, congo red, etc.

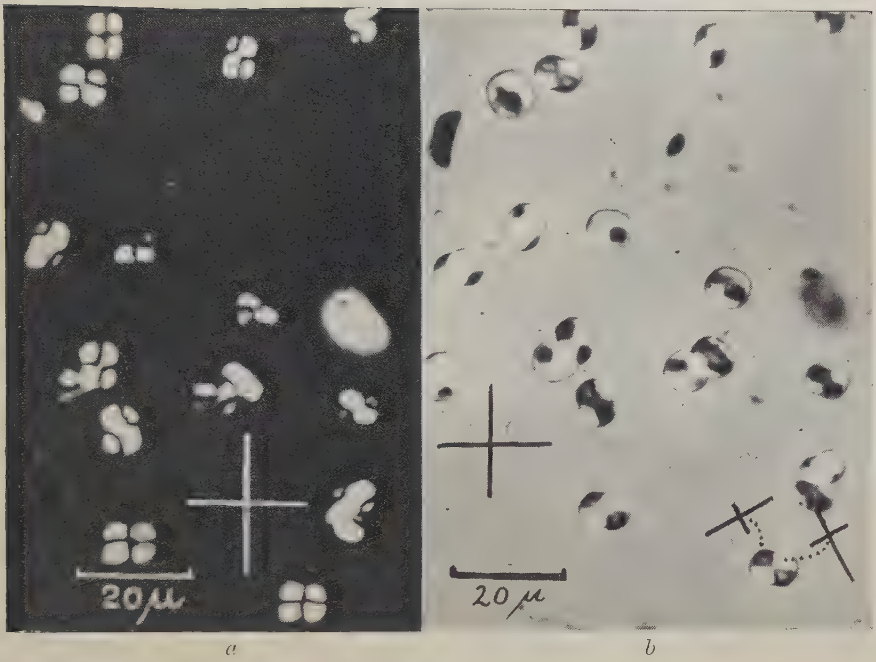

Fig. 1. Chitosan sulphate sphaerocrystals from baker's yeast.

*a.* Between crossed nicols (vibration planes indicated). Note symmetric Brewster cross of flat lying platelets and distorted cross of upright ones;

*b.* The same crystals as in *a* between crossed nicols, but with compensated double refraction in two quadrants. Orientation of major refractive index indicated. Note circular form of flat lying crystals and 8-shaped side view.

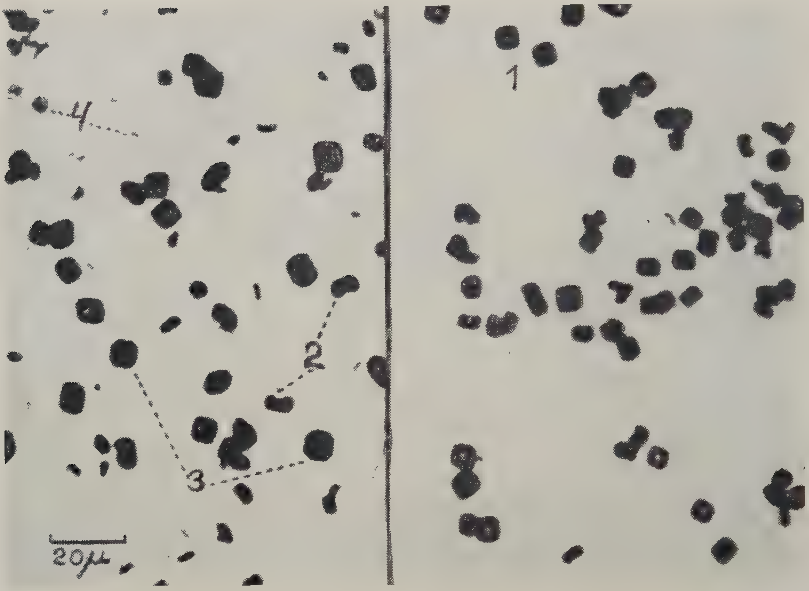

Fig. 2. Tetragonal (1) and octogonal (3) chitosan sulphate sphaerocrystals stained with acid fuchsin. Note the dumbbell-shape of large crystals on side view (2) and the flatness of the small ones (4).

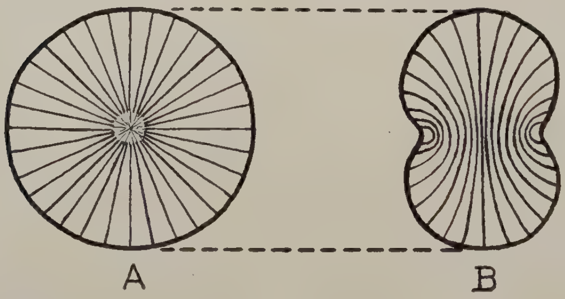

Fig. 3. Orientation of crystallites (and molecules) in chitosan sulphate sphaerocrystals. *a.* Top view; *b.* Side view.

In our opinion, therefore, the distrust in the chitosan sulphate test as expressed in literature, is unjustified. The chitosan sulphate method shares simplicity with the chitosan-iodine method, but is more sensitive and completely reliable. However, even a consistently negative result, obviously does not prove that traces of chitin are absent, since all methods have a sensitivity limit. As appeared from experiments with X-ray diffraction [(10) and forthcoming publication] and paper chromatography (§ 4) these methods need more material and more preparative chemical work to reach the same level of sensitivity.

## § 4. CHROMATOGRAPHIC EXAMINATION OF YEAST CELL WALL HYDROLYSATE.

As remarked in § 1, SALKOWSKI and MEIGEN and SPRENG reported mannose and glucose to be the main component radicals of the cell wall residue of baker's and brewer's yeast. However, ZECHMEISTER and TÓTH and HASSID *et al.* in vain tried to find mannose and like BELL and NORTHCOTE, stated glucose to be the sole component radical.

The last mentioned authors hydrolysed the cell wall residue of baker's yeast and analysed the sugars in the solution by filter paper chromatography. Although the Rf values of glucose and mannose differ only a little, mannose, if present in the amount reported by MEIGEN and SPRENG, presumably should have been detected as a separate spot. Moreover, a spot of the main product of acid hydrolysis of chitin, *viz.* glucosamine, should also have appeared with the ammoniacal $AgNO_3$ reagent, used to reveal the location of sugars, unless the hydrolysate introduced on the paper had been too dilute. Only a glucose spot is reported to be present, but the absence of mannose and glucosamine is not specially mentioned.

It seemed worth while, therefore, to repeat the chromatographic examination of the yeast cell wall hydrolysate, using more concentrated solutions and paying special attention to mannose and hexosamines.

Thirty g baker's yeast was cleaned by boiling 3 × 2 hrs in 3% NaOH and 3 hrs in 2% HCl. The washed and dried residue (0.4 g), consisting of cell walls still enclosing one or two granules of unknown composition, was kept in 5 ml 72% sulfuric acid (w/w basis) at 20° C. for 3 days. After adding 50 ml water and boiling

8 hrs, the hydrolysate was neutralized with $BaCO_3$, filtered, dried in a warm air current and dissolved in 2 ml water. About 0.005 ml was introduced onto a strip of Whatman nr 1 filter paper, which subsequently was irrigated downward for 3 days at 22° C. with the butanol-acetic acid-water solvent as used by PARTRIDGE (17). Glucose, mannose, glucosamine hydrochloride and hydrolysed lobster chitin were used as references.

On revealing the separated constituents by spraying either ninhydrin or ELSON and MORGAN's hexosamine reagent (17) or appropriate sugar reagents, a faint glucosamine spot and a very faint mannose spot appeared, in addition to a very strong one, corresponding with glucose. The presence of glucose depressed the Rf values of glucosamine.

A much stronger glucosamine spot, resulting from yeast chitin, could be obtained by extraction of the usual yeast cell wall residue with cold 30% HCl and precipitation of the dissolved fraction by neutralisation with dilute alkali, followed by hydrolysis and partition chromatography.

The presence of a trace of mannose in the hydrolysate of the baker's yeast cell wall residue as used by us, might be ascribed either to remnants of the so-called yeast gum, which has been found to consist mainly of a mannan, or to the incorporation of a small amount of mannose radicals in the cell wall glucan.

## Summary.

According to literature a glucan is the main cell wall constituent of baker's yeast and probably of all yeasts. Chitin is reported in some filamentous *Endomycetaceae,* but is said to be absent in all other yeasts tested.

A modified chitosan sulphate crystallization test for detecting chitin in yeasts and the relevant characteristics of these crystals were described. It was applied to 29 yeast species in addition to baker's and brewer's yeast. Except *Schizosaccharomyces octosporus* all were found to contain chitin as a cell wall constituent. However, the amount was very small, varied in different species and seemed to increase in older cultures.

Chitin has also been demonstrated as glucosamine on paper chromatograms of concentrated hydrolysates of the cell wall residue, obtained after treating baker's yeast with boiling dilute alkali and acid.

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# THE PRODUCTION OF HOMOGENTISIC ACID OUT OF PHENYLACETIC ACID BY *ASPERGILLUS NIGER*

by

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## 1. INTRODUCTION.

In the first chapter of his book "Chemical activities of fungi" FOSTER (2) remarks that the application of mould metabolism seems to offer "as yet unexplored potentialities in the field of industrial fermentations", in so far as attention should be given to substrates other than carbohydrates. It is, indeed, well-known that in the industrial preparation of the main fungal products, such as gluconic acid, citric acid, and itaconic acid, always carbohydrates are used as starting material. With a view to the experiences obtained with incomplete oxidations brought about by bacteria — which oxidations often lead to very specific products configurationally related to the oxidation substrates — one could therefore expect a similar behaviour in mould metabolism.

In this trend of thought we have deemed it worth-while to give once more attention to the possible occurrence of incomplete oxidation products in cultures of *Aspergillus niger* using various fatty acids and dicarboxylic acids as sole source of carbon.

We shall refrain from giving here a detailed report of the outcome of these investigations which on the whole were unsatisfactory. Both in surface and in shake cultures the growth of the fungus was usually rather restricted and no characteristic oxidation products could be detected. Also a reduction of the oxygen tension in shake cultures, by leading nitrogen with a few percent oxygen through the vessels, did not improve the situation. For these reasons we decided to apply the replacement culture technique according to which first fully-grown mould pads are prepared by growing the fungus on a carbohydrate containing medium, after which the liquid beneath the pad is drawn off and replaced by sterile water. Then this wash water too is removed, and substituted by an aqueous solution of the substrate to be tested. In this way the substrate is subject to the metabolic activity of the preformed mould pad, and this under conditions of restricted aeration which may be expected to favour incomplete oxidation.

However, also the results of a rather extensive series of experiments along this line were disappointing: notwithstanding a great variation in the substrates tested no specific oxidation products could be observed. Apart from the carbon dioxide formed, oxalic acid was the only oxidation product which regularly was encountered.

This negative result induced us to extend our investigation to aromatic acids in the hope that the presence of the benzene ring might counteract the conversion of the side chain into oxalic acid. The first experiences were not encouraging, since under the experimental conditions chosen both benzoic- and salicylic acid were also quite rapidly consumed with ample production of oxalic acid.

The next compound tested was phenylacetic acid, and here it was at once evident that the oxidation proceeded in a somewhat particular way. The medium soon turned dark brown, and a preliminary analysis showed that next to the oxalic acid one or more compounds were present which brought about a strong reduction of Fehling's solution.

In the following chapters we shall report on a detailed study of the oxidation of phenylacetic acid which led to the isolation of homogentisic acid as a product of mould metabolism.

## 2. EXPERIMENTAL TECHNIQUE.

The strain of *Aspergillus niger* used in these experiments had been isolated from an air contamination on some agar medium. During the investigation the strain was kept on Czapek-Dox agar medium containing: 3 % saccharose, 0.2 % $NaNO_3$, 0.05 % KCl, 0.05 % $MgSO_4$ 7 aq., 0.001 % $FeSO_4$ 7 aq., 0.1 % $KH_2PO_4$, 2 % agar in tap water, adjusted to pH 6.5.

Erlenmeyer flasks of 500 ml containing 100 ml of Czapek-Dox liquid medium (pH = 4.5) were inoculated with about 0.5 ml of a spore suspension of the fungus. In order to obtain a more or less homogeneous suspension a few ml of a 1 : 10.000 solution of sodium laurylsulphonate were brought into a good sporulating culture tube, after which the conidia were evenly distributed in the liquid with the aid of a nichrome loop.

After incubation at 30° C. for 2 or 3 days a cohaerent mould mat had been formed in the Erlenmeyer flasks. Then the culture fluid was gently poured out, and subsequently the lower surface of the mould pad was repeatedly washed with sterile water.

In the meantime the pH of a 3 % solution of phenylacetic acid had been adjusted to about 7.0 with caustic soda. After sterilisation 100 ml of this solution was carefully brought under the pad. Then incubation was continued for 5 to 9 days. As has already been remarked, during this period the medium turned dark brown, and obtained marked reducing activity. The pH had then dropped to 4.0—4.5.

### 3. IDENTIFICATION OF OXIDATION PRODUCTS.

In some preliminary experiments it was established that the reducing activity of the medium towards FEHLING's solution attained a maximum in an average period of about 7 days, after which this activity slowly decreased. Since the appearance of a reducing compound in the medium was quite unexpected, we decided to analyze the medium at the stage of maximum concentration of this compound. At this time it was evident that the phenylacetic acid had almost completely disappeared from the medium. It was further found that a non-negligible part of the acid had been converted into oxalic acid, and this oxidation product was first removed by adding a sufficient amount of a 20 % solution of $CaCl_2$ to the culture medium. We have refrained from a preliminary neutralization of the medium, since the calcium oxalate precipitated in a weakly acid medium can much more easily be separated from the liquid either by filtration or centrifugation. Moreover, it was found that neutralization of the medium was undesirable, because this led to a marked increase of the brown coloration of the medium.

It appeared that after the removal of the oxalic acid with calcium chloride the reducing activity of the remaining liquid was unimpaired. The liquid was then concentrated in vacuo to $^1/_5$ of its original volume. It was then attempted to extract the reducing compound from the concentrated aqueous solution with various organic solvents, and it was found that, indeed, this compound could be dissolved in ether. In addition it was established that ether did not extract the reducing compound when the aqueous solution had been neutralized beforehand, which, of course, gave a strong indication for the acidic character of the compound in question.

In the later experiments the aqueous concentrate was acidified with some drops of N HCl, using congo red as an indicator, and then extracted with ether in a percolator until the aqueous phase had practically lost its reducing activity which usually took 10—12 hours.

It was probable that the slightly brown coloured ether extract would still contain some unaltered phenylacetic acid, and therefore a separation of this acid from the reducing compound was desirable. This proved to be possible in the following way. The ether was distilled off and the residue dissolved in a small quantity of water, after which the solution was extracted with benzene in a separating funnel. If, indeed, any phenylacetic acid has remained, it is then dissolved in the benzene, whilst the reducing compound practically remains in the aqueous layer.

It was then found that addition of a saturated solution of lead acetate to the aqueous solution led to a precipitate in which microscopically brown needles could be detected. Moreover, when these needles were brought into a dilute solution of silver nitrate immediately reduction to metallic silver occurred, proving that the crystalline product was connected with the reducing principle. In further experiments it was found that a nice crystalline precipitate of the lead compound was obtained when the aqueous solution was first heated to boiling temperature. The lead salt was recrystallized from hot water, using some norit, and in this way a satisfactory yield of colourless needles was obtained.

In order to eliminate the lead from this compound the needles were mixed with a suitable quantity of sodium bisulphate and ground to a homogeneous powder in a mortar. The fine mixture was extracted with (undried) ether, the etheral solution dried with anhydrous $Na_2SO_4$ and evaporated to dryness. In this way a fine crystalline yellowish product was obtained.

This product proved to have a melting point of 145—146° C. and the following properties. Addition of some drops of a solution of $FeCl_3$ to the aqueous solution gave a rapidly vanishing blue-green coloration. Even a slightly acid solution of the compound immediately brought about a strong reduction of an aqueous solution of silver nitrate. Also FEHLING's solution was reduced in the cold.

An elementary analysis performed in the Laboratory of Organic Chemistry of the University yielded the following results:

40.05 mg of the compound gave 17.6 mg $H_2O$ and 83.9 mg $CO_2$
39.85 mg of the compound gave 17.3 mg $H_2O$ and 83.6 mg $CO_2$

| | % C | % H |
|---|---|---|
| Found | 57.1 | 4.9 |
| Found | 57.2 | 4.8 |
| Calculated for $C_2H_2O$ | 57.14 | 4.76 |

The determination of the equivalent weight of the compound by microtitration with 0.05 N alkali, using phenolphthaleine as an indicator gave as a result: 164. Assuming the compound to be a monobasic acid the molecular weight is therefore 164 which then would indicate a molecular formula of $(C_2H_2O)_4$ (Mol. Wt. = 168).

The complex of all data available made it at once probable that we were dealing here with a dihydroxyphenylacetic acid. A study of the various isomers described showed that only the melting point of 2,5-dihydroxyphenylacetic acid (146—147° C. according to BEILSTEIN) closely approximated the melting point of our compound. The biochemical significance of this isomer — better known as homogentisic acid — in animal metabolism added to the probability of the identity.

It was, therefore, tempting to compare our preparation with some homogentisic acid isolated from a case of alcaptonuria. Through the kind intermediary of Dr C. M. VEENHUYSEN at the Hague we succeeded in obtaining urine samples of an elderly female patient. As determined with the method of BRIGGS the urine contained 1.4 mg homogentisic acid per ml. Ether extraction and purification of the lead salt finally yielded a crystalline preparation of the acid.

In the meantime we had also requested a sample of homogentisic acid from Dr A. NEUBERGER of the National Institute for Medical Research in London who in recent years has made extensive studies on its occurrence in human metabolism. Dr NEUBERGER was so kind as to comply with our request for which we offer him our most sincere thanks.

It was found that, indeed, both a mixture of Dr NEUBERGER's and of our own mould preparation, and a mixture of the latter with our own alcaptonuria preparation again melted in the neighbourhood of 146° C., whilst all these preparations were found to behave similarly in other respects tested.

Herewith proof was given that under certain conditions in the oxidation of phenylacetic acid by *Aspergillus niger* homogentisic acid is formed.

Before discussing the significance of this finding we shall first briefly report on some additional experiments dealing with the formation and consumption of this metabolic product.

## 4. SOME ADDITIONAL OBSERVATIONS ON THE PRODUCTION AND CONSUMPTION OF HOMOGENTISIC ACID.

The identity of the reducing oxidation product once being established, it was desirable to collect some quantitative data regarding the production and consumption of the homogentisic acid.

Until now the quantity of the unknown reducing compound present in the medium had been estimated with the aid of FEHLING's solution using BERTRAND's oxidimetric modification of FEHLING's method. We now decided to estimate the homogentisic acid with the aid of the well-known method of BRIGGS (1) [1].

For the determination we proceeded as follows: 1 ml of the culture medium was diluted to 25 ml. From this dilution 5 ml were brought into a volumetric flask of 25 ml. Then 2 ml of a 5 % solution of ammonium molybdate in 5 N $H_2SO_4$ and also 2 ml of a 1 % aqueous solution of $KH_2PO_4$ were added. Subsequently the volume was made up to 25 ml. After one hour standing the intensity of the blue-green colour developed was compared in a DUBOSCQ colorimeter with a standard colour obtained by subjecting 1 ml of a solution containing 350 $\gamma$/ml of pure homogentisic acid to the molybdate reaction.

We then convinced ourselves that in the dilution applied neither the brown pigment present in the medium, nor the oxalate formed interfered with the determination.

Using this method we found in various experiments that the maximum amount of homogentisic acid formed in the medium containing 3 % of sodium phenylacetate varied between 2.3—5.0 mg/ml, indicating a 7.2—15.7 % molecular conversion of the phenylacetic acid.

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[1] It is true that NEUBERGER (4) in his recent studies gives preference to a method newly developed by him in which silver nitrate is reduced at a pH = 4.4. NEUBERGER shows that BRIGGS' method is less specific, because other p. dihydroxyphenyl derivatives also act on BRIGGS' reagent. In our case BRIGGS method seemed, however, sufficiently reliable and was chosen for the sake of simplicity.

It can be added that besides the homogentisic acid probably also small quantities of its direct oxidation product *viz.*, benzoquinone-acetic acid were always present, as judged by the oxidation of KI in strong acid solution (cf. NEUBERGER (4)).

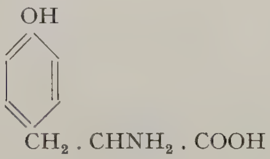
In all experiments also oxalate could be detected in the medium, the final quantities varying between 8—11 mg/ml. No further oxidation products could be found so that the conclusion is warranted that the greater part of the phenylacetic acid had been completely oxidized to carbon dioxide and water.

Altogether it seemed probable that the homogentisic acid is a normal intermediate product in the oxidation of phenylacetic acid by *Aspergillus niger* and is ultimately converted into oxalic acid and carbon dioxide. In order to strengthen this hypothesis an experiment was made in which a 3 % solution of sodium homogentisate (pH = 6.5) was brought under a fresh mould mat. After 7 days the medium had again become brown, and the homogentisic acid had practically disappeared. Besides oxalic acid — about 10 mg/ml — no other oxidation products could be found.

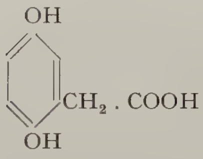
## 5. DISCUSSION OF THE SIGNIFICANCE OF THE PRODUCTION OF HOMOGENTISIC ACID.

At the time when these experiments had been completed no references to the occurrence of homogentisic acid in microbial metabolism were known to us. On the other hand this acid has already for a long time played an important rôle in human and animal metabolism. Its regular occurrence in the urine in cases of the metabolic disorder known as alcaptonuria — in which the urine turns dark brown — has been proved ever since WOLKOW and BAUMANN (9) in 1891 isolated the acid from the urine of a patient and showed that this compound was the mother substance for the formation of the brown pigment. These early workers already speculated on the origin of this till then quite unknown compound, and came to the logical conclusion that it might well originate from the only aromatic amino acid known at that time, *viz.* tyrosine, this compound being a regular component of all food proteins. In accordance herewith they demonstrated that an addition of free tyrosine to the food, indeed, led to an increased excretion of homogentisic acid by the patient. Later investigators established that the same effect was obtained, if phenylalanine was given instead of tyrosine, all evidence available being in favour of the view that in these cases the phenylalanine is primarily oxidized to tyrosine (cf. Moss and Schoenheimer (3)). In a more recent period an interesting development took place in so far as Papageorge and Lewis (5), Sealock *et al.* (7) and various other American authors have shown that an experimental alcaptonuria can be developed in normal rats and mice by feeding these with large amounts of phenylalanine, and also in guinea pigs, in as far as the food is deficient in vitamin C.

A remarkable aspect of the conversion of tyrosine into homogentisic acid is that this implies a shift in the position of one of the substituting groups as present in the tyrosine. This is at once clear from the formulae:



tyrosine



homogentisic acid

It is worth-mentioning that this rather startling feature of tyrosine oxidation was already clearly recognized by the discoverers of homogentisic acid, Wolkow and Baumann (9). Their comment is too interesting not to be cited here:

> „Würde in den Organen und Geweben des Körpers als solchen die Umwandlung des Tyrosins zur Homogentisinsäure bewirkt, so wäre damit bewiesen, dass aus einem Benzolderivat von bestimmter Constitution jede andere aromatische Verbindung, welche in keinerlei erkennbarem chemischem Zusammenhang zum ersteren steht, gebildet werden kann. Wenn diese Voraussetzung zuträfe, so würde durch diese einzige Thatsache der sichere Boden, welcher durch die chemische Erforschung der Einwirkung des thierischen Stoffwechsels auf viele Hunderte von organischen Verbindungen in den letzten Jahrzehnten geschaffen worden ist, verloren gehen. Da in solchem Falle Alles aus Allem durch den Stoffwechsel gebildet werden könnte, würde die Untersuchung einzelner chemischer Vorgänge des Lebensprocesses so gut wie gegenstandslos werden."

Their escape from this difficulty is after all quite simple. Unwilling to ascribe such an "immoral" biochemistry to the animal body, they take refuge in making micro-organisms present in the colon responsible for it! It should be added, however, that their attempts to detect homogentisic acid in the faeces were unsuccessful, nor could this compound be found when tyrosine and water were added to the faeces and the mixture was incubated for some days at blood temperature.

Later investigators of animal metabolism have not had the same objections against the idea that animal tissues would be able to bring about such a shift in the position of one of the substituents in the benzene ring, more particularly in that of a side chain. This is partly due to the fact that in 1903 BAMBERGER described an analogous shift in the purely chemical oxidation of p-hydroxytoluene with CARO's reagent. Moreover, in later years it has been found that homogentisic acid also arises in the *in vitro* oxidation of tyrosine under the influence of rat liver tissue. That under these conditions a position shift of the side chain indeed occurs has been definitely proved with the aid of the radioactive carbon isotope in the beautiful investigation by SCHEPARTZ and GURIN (6).

In the meantime the suggestion made by WOLLOW and BAUMANN in 1891 that micro-organisms might be able to produce homogentisic acid has remained without response for almost sixty years.

In this connection it can be remarked that after having identified homogentisic acid as an intermediate product in the oxidation of phenylacetic acid by *Aspergillus niger*, we have at once made some experiments in order to establish whether under identical conditions homogentisic acid is also formed in the oxidation of tyrosine and of phenylalanine by this mould. Strong indications that this, indeed, is the case have been obtained: also with these substrates the medium turns brown and a reducing compound of the homogentisic acid type is formed. However, owing to the fact that only small quantities of the said substrates were available to us, the isolation of the homogentisic acid in pure state has not yet been successful.

In this stage of our investigation it came to our knowledge through Chemical Abstracts of January 1951 that the formation of homogentisic acid from tyrosine by *Aspergillus niger* has already been reported in 1950 by the Russian investigator UTKIN (8). In this study the experimental conditions are rather different from those applied by us: UTKIN isolated homogentisic acid from cultures in which the mould grew in a medium containing 4 % glucose and 0.5 % tyrosine as sole source of nitrogen.

Anyhow, the biochemical conversion of phenylacetic acid into homogentisic acid, as described in the present paper, has to our knowledge not yet been reported before.

We must leave to the isotope workers to answer the question in how far also in this oxidation a shift in the position of the side chain occurs. In favour of this assumption is that the analogous oxidation of phenylalanine has been shown to start with the introduction of a hydroxyl group at the 4-place, but on the other hand the possibility cannot be excluded that in the case of phenylacetic acid we are dealing with a direct successive oxidation at the 2- and 5-places.

## Summary.

With the aid of the replacement culture technique it was found that a strain of *Aspergillus niger* was able to produce homogentisic acid out of phenylacetic acid with a yield upto 15.7 % of the theoretical amount.

Evidence is in favour of the view that homogentisic acid is a normal intermediate in the oxidation of the said substrate.

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(From the Botanical Laboratory, University of Pennsylvania, Philadelphia).

# METABOLISM AND MORPHOGENESIS IN A NEW *BLASTOCLADIELLA*

by

**EDWARD C. CANTINO** [1])

(Received May 16, 1951).

## 1. INTRODUCTION.

In 1949, a species of the aquatic Phycomycete, *Blastocladiella,* was discovered in the fresh-water pond adjacent to the Botanical Laboratory of the University of Pennsylvania and a single-spore isolate was subsequently obtained in pure culture. The fungus is heterogamous (size and color differences) and possesses many characteristics of the sexually-reproducing *B. variabilis* and *B. stübenii* and asexually-reproducing *B. simplex*; it has not yet been designated a new species. The following report concerns: (1) a biochemical interpretation of morphogenesis, based upon the nature and function of internal and external factors (with emphasis upon metabolism and the tricarboxylic acid cycle) effecting the formation of resistant sporangia, and (2) an investigation of environmental factors involved in a two-step germination mechanism in resistant sporangia.

In view of the timely appearance of EMERSON's (11) review, and of the taxonomic treatments of COUCH and WHIFFEN (9) and SPARROW (33), the reader is referred to them for the general morphology, modes of reproduction, and phylogenetic importance of the genus.

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[1]) The greater part of this work was completed during the tenure of a Guggenheim Fellowship at the Laboratory of Microbiology, Technical University, Delft, Holland. It is indeed a great pleasure to acknowledge the kind and generous hospitality of Professor KLUYVER and all of the members of his laboratory; to them, the writer is sincerely indebted for a very pleasant and profitable year in Delft.

## 2. MATERIALS AND METHODS.

Because of the variety of experiments performed, only general methodology follows; specific details are found in the context.

The fungus was accidentally "trapped" on silica gel in Petri dishes which had been submerged by Mr. JOSEPH DEVLIN (Botanical Laboratory University of Pennsylvania) in the fresh-water pond. The ensuing growth was practically uni-fungal, thus facilitating single-spore isolations by the methods used for *Blastocladia* (EMERSON and CANTINO (12)).

The various media used were: (1) water-hempseed medium (cf. EMERSON (12)). (2) medium P: 10 g peptone (a non-standardized product), 5 g NaCl, and 20 g shredded agar dissolved in 4 liters tap water, neutralized to pH 7.3, filtered, and autoclaved 15 min. at 120° C. (3) medium B: 1.4 g $KH_2PO_4$, 0.6 g $Na_2HPO_4$, 0.2 g $MgSO_4 . 7H_2O$, 1.0 g Difco vitamin-free Casamino acids, 20 g Difco Nobles agar (purified by pyridine- and alcohol-extractions) dissolved in one liter distilled water and autoclaved 15 min. at 110° C.

The *a*-ketoglutaric acid was a recrystallized student preparation, the vitamins and amino acids were Merck and G.B.I. products, and sodium pyruvate was from Hoffman–La Roche. *Cis*-aconitic acid, prepared from *trans*-aconitic (E. Merck, Darmstadt) by the method of UMBREIT *et al.* ((35) p. 185—186), was kindly supplied by Dr B. J. D. MEEUSE, Laboratory for Technical Botany, University of Delft.

Thiamine assays were made with the Delft strain of *Pichia Dombrowskii* (Sachetti) no. 9.10.1 (cf. BURKHOLDER (5)), and lactic acid was determined according to FRIEDEMANN and GRAESER (16).

With few exceptions, plates were "streaked" with spore suspensions by placing an aliquot (usually 0.1 cc) in the center of the plate, tilting to cause the drop to move a distance of approximately 4 cm, reversing the tilt and thus forcing the liquid for 4 cm in the other direction, and finally establishing a second streak at right angles with the first and through its center by tilting at the appropriate angle. This insured a reasonably constant streak diameter and, because of spore motility, a constant population density for any given set of replicate experiments.

For storage purposes, resistant sporangia harvested from solid media were sometimes transferred to sterile strips of filter paper as EMERSON (10) did for *Allomyces*. Occasionally, hemp seeds bearing hundreds of sporangia were transferred *in toto* to sterile test tubes lined with filter paper (for rapid absorption of surface water from the seed and fungus).

Moisture chambers were essentially similar to those used by SONNEBORN (31).

## 3. THE FORMATION OF RESISTANT SPORANGIA (R.S.).

Because it was of utmost importance to achieve control over this phase of morphogenesis in the life cycle of *Blastocladiella* spec., and because sexual fusions were apparently not involved (CANTINO (8)), attempts were made to evaluate the relation of environmental factors to R.S. formation. When R.S. spores are streaked on medium P, over 99 % of the plants formed are almost invariably gametophytes, R.S. plants appearing very infrequently¹). Following maturation of the gametophytes (*ca.* 24 hours at 30° C., or 48 hours at 20° C.), gametes are discharged, even on very dry plates, but are localized over an area (*ca.* 250 to 700 $\mu$ diam.) immediately adjacent to the parent thallus. The number of gametes discharged is variable but the vast majority can usually be seen moving about in the minute volume of exudate liberated with the gametes; many may remain trapped within the gametangium if the exit pore is blocked by previously discharged gametes or if the agar surface is sufficiently dry to rapidly reabsorb part of the exudate. Thus, a cluster, as it will hereafter be called, is formed. Such clusters invariably turn brown due to direct parthenogenetic development of R.S. plants from the original gametes and their progeny. They contain variable proportions of colorless plants which cease growth prematurely, thalli bearing mature deeply-pigmented R.S. with great variation in size (15 × 20 to 165 × 170 $\mu$; Fig. 3), and intermediate types at different stages of development. Localized nutrient deficiencies and differential accumulations of metabolic products in the cluster, as well as the purely physical "crowding" of the plants, must play a part in this variability. Mature R.S. (Figs. 1 and 2) possess a thick (usually 2 to 3 $\mu$), brown, obviously-pitted wall, often-conspicuous oil globules, and discrete linear surface markings (similar to those of *B. simplex*) which most often appear to be depressions bordered by raised areas. Many of the smaller sporangia, however, show different quantities

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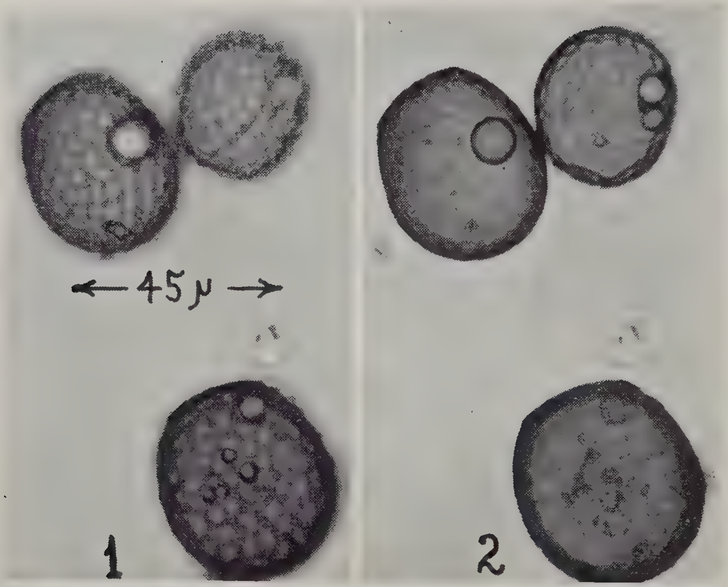
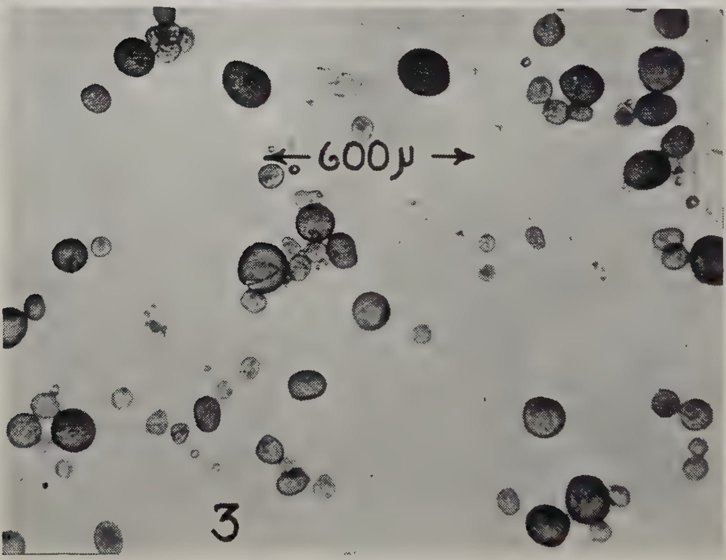
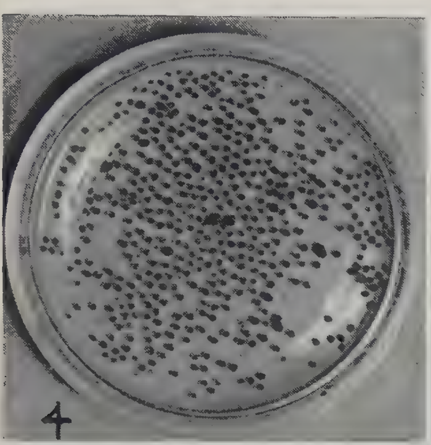
¹) STÜBEN (34) also noted the rarity with which individual plants bearing R.S. appeared in *B. stübenii*.

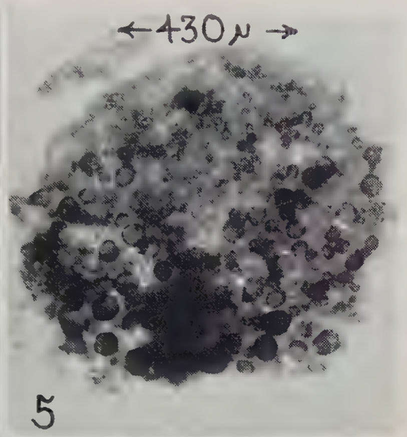


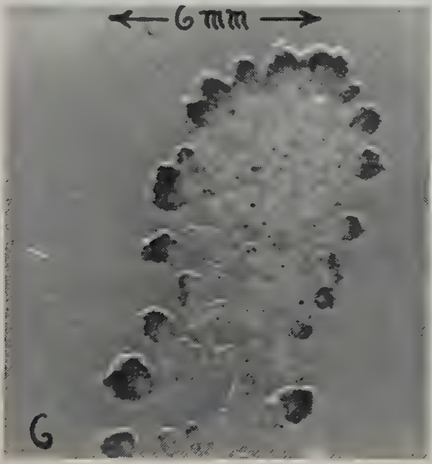


Figs. 1 and 2. Surface view and median optical view, respectively, of rather small but mature resistant sporangia scraped from the surface of a 10 day old cluster on medium P at 20° C. Note thick, pitted wall, linear surface markings, and oil globules.

Fig. 3. Resistant sporangia removed at random from the surface of a 12 day old cluster on medium P at 20° C. Note size variation.







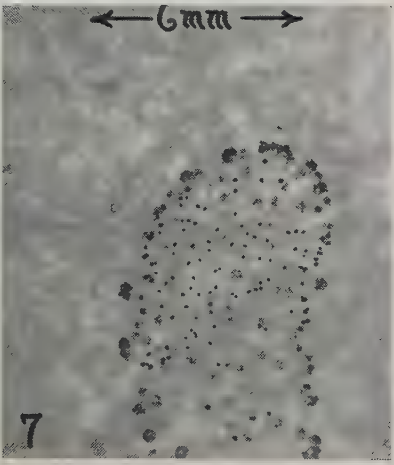
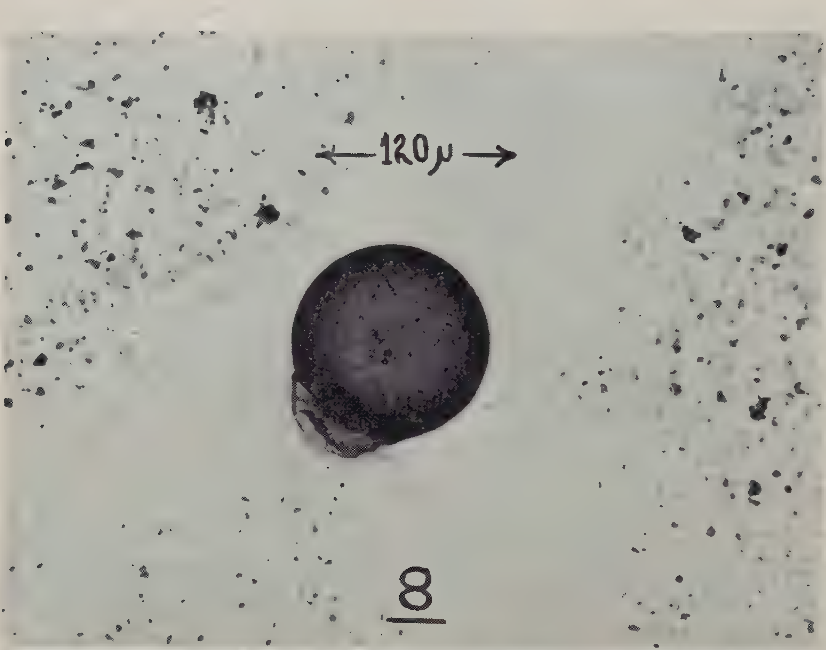


Fig. 4. Resistant-sporangial spheres removed from 10 day old shake cultures (liquid medium P) at 30° C. × *ca.* 1.

Fig. 5. A peripheral cluster (15 days old) from the edge of a streak on medium P at 20° C. Note local concentration of larger R.S. in region (bottom) farthest removed from central axis of streak.

Fig. 6. The tip of a streak (18 days old) on medium P at 20° C. Note the more rapid rate of growth and associated pigmentation of edge clusters, and the absence of isolated R.S. plants.

Fig. 7. The tip of a streak (6 days old) on medium P plus M/100 $NaHCO_3$ at 20° C. Note the *ca.* 50% incidence of individual R.S. plants from R.S. spores, and the relative localization of young clusters along the edge.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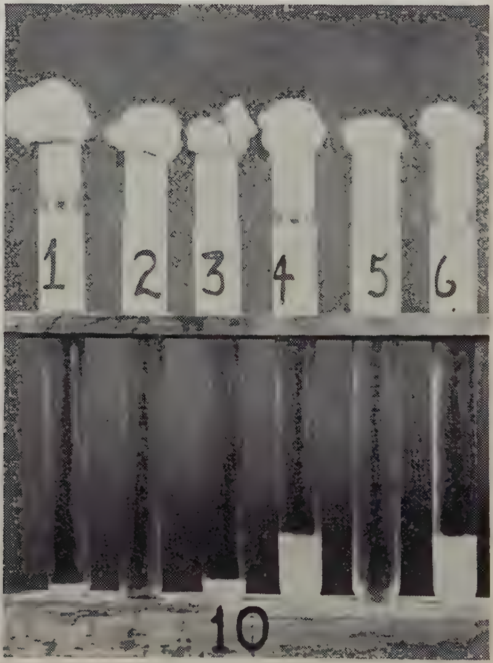
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of oil globules and different degrees of pigmentation, from very pale yellow to dark brown. Pitting, too, is variable; completely smooth walls, irregular striations (really depressions, which sometimes appear to be confluent pores in series), and discrete pits may all be found among the R.S. of a single cluster.

In well-populated streaks, the largest, most uniformly pigmented clusters occur along the edge, those centrally located being smaller in diameter and bearing fewer dark brown sporangia (Fig. 6). Furthermore, in peripheral clusters, the greatest number of large, deeply pigmented R.S. plants are localized on the side farthest removed from the central axis of the streak (Fig. 5).

This behavior of the gametophytes appears to be duplicated in liquid media. In shake cultures, a suspension of spores or gametes gives rise in 2 or 3 days at 30° C. to compact dark brown spheres, each composed of hundreds of R.S. plants entangled with one another by their rhizoids and elongated thalli (Fig. 4). This is supposedly due in part to a relative lack of gamete discharge from the parent gametophytes with subsequent germination *in situ* among some of the 2nd., 3rd., etc. generations.

Clearly, the objective was to grow single, isolated, R.S. plants of uniform size and composition under precisely controlled conditions. Carbon dioxide and pH were limiting factors in the formation of resistant sporangia of the close relative *Blastocladia* (EMERSON and CANTINO (12); CANTINO (6)), and were therefore tested with *Blastocladiella*. Though pH was not critical, $CO_2$ was indeed of fundamental importance in determining whether or not R.S. plants or gametophytes were formed from spores. Over 95%

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Fig. 8. A representative R.S. plant (8 days old) from medium P plus 0.5% $CaCO_3$ at 20° C. The basal stalk, damaged in this specimen due to an adjacent fissure in the agar, is always longer and more prominent than is evident here.

Fig. 9. The tip of a streak (30 days old) on medium B plus 0.5% $CaCO_3$ and $2.5 \times 10^{-3}$ M *trans*-aconitate at 20° C. Note the *ca.* 100% incidence of R.S. plants and the localization of the 2 sole clusters along the edge.

Fig. 10. The total amount of growth at *ca.* 20 to 22° C. in representative cultures (from sets in triplicate) on $B_1$-deficient media. All flasks contained 100 cc medium B plus 3.0 g glucose per liter, in which $2.5 \times 10^{-2}$ M $NaHCO_3$, $1.9 \times 10^{-3}$ M Na acetate, 200 $\mu$g pantothenate and 50 $\mu$g thiamine per liter were incorporated as follows: (1) $NaHCO_3$ and Na acetate. (2) Na acetate. (3) $NaHCO_3$. (4) $NaHCO_3$, Na acetate, and pantothenate. (5) no additions. (6) thiamine.

of them may give rise directly to R.S. plants on medium P containing 0.5 to 1.0% calcium carbonate (Fig. 12). Spores behave similarly in the presence of $NaHCO_3$ (Fig. 11). Gametes from 1st. generation gametophytes are induced to develop parthenogenetically into R.S. plants in precisely the same way! Thus, under these conditions, the life history represents a simplified *Brachyallomyces* cycle: R.S. plant → R.S. spore → R.S. plant, etc; this phase of the work, however, will be amplified elsewhere (CANTINO (8)). Even with maximum production of R.S. plants, a few spores may form gametophytes with subsequent production of clusters, but these are most often restricted to the edge of the streak, and usually at its tip; the fact that they are the farthest removed from the population center may be a reflection of differences in available nutrients or accumulated metabolic products. [cf. Fig. 9, in which over 95% incidence of R.S. plants, induced with *trans*-aconitate (see later), is representative of the situation found on carbonate and bicarbonate at optimum concentrations]. At somewhat lower $NaHCO_3$ levels, when fewer R.S. plants are produced, clusters continue to predominate along the edges; but, as it is decreased further, they are dispersed with greater frequency among the R.S. plants in the central areas of the streak (Figs. 7 and 8). In spite of the striking effect of such salts, inconsistent results were obtained with plates placed in gas chambers containing different quantities of $CO_2$. Above 5% $CO_2$, growth did not occur; below this value, the incidence of R.S. plants seldom reached 50%.

Plates of medium P were also covered with 0.3 to 0.5 cm layers of bicarbonate solutions of different concentrations some 8 hours after inoculation; e.g., after the rhizoidal system of the young germling was insured a secure foothold on the agar surface. After correcting for dilution by the agar, results were roughly comparable to those shown in Fig. 11. In fact, spores transferred to liquid medium P containing different concentrations of $NaHCO_3$ behaved similarly. In these instances, at optimum bicarbonate concentrations, R.S. plants were clearly visible to the naked eye as small brown bodies firmly attached to the bottoms of the flasks. A few gametophytes were always formed, however, and even when they represented a very small fraction of the population, subsequent discharge of gametes from them soon increased the number of free-floating plants sufficiently to obscure the initial observations. To insure that it was not merely a general "salt effect", $MgSO_4$, $NaNO_3$, KCl, and NaCl were tested in liquid and solid media at similar concentrations, but R.S. plants were not produced.

A relatively small increase in available $CO_2$, then, appeared to be necessary for the formation of individual R.S. plants. This was tentatively reconciled with the appearance of large quantities of R.S. in the second-generation clusters on agar by assuming that a local increase in available $CO_2$ above that in the immediate surroundings existed among the densely-packed plants. It had been determined, however, that resting "cell suspensions" in fermentometers (containing medium B plus 3.0 g glucose and 50 $\mu$g thiamine per liter) formed no detectable $CO_2$. Three flasks, containing 500 cc of liquid medium P and fitted for continuous $CO_2$-free aeration, were inoculated with spore suspensions, and total $CO_2$ evolved during growth was determined by absorption in alkali and subsequent titration. The fungus was harvested after *ca.* 2/3 of the growth period was completed, and dry weights were obtained by heating for 24 hours at 98° C.; $6.9 \times 10^{-4}$ millimoles $CO_2$ per mg dry weight of *Blastocladiella* were formed, a rather small quantity indeed. The following calculations were then made: From Fig. 11, the minimum concentration of $NaHCO_3$ yielding maximum numbers of R.S. plants was *ca.* M/75. From earlier determinations, the weight of an average young cluster was estimated to be $10^{-2}$ to $5 \times 10^{-2}$ mg; the volume of agar over which rhizoids extend was roughly equivalent to a block 2 mm square and 1 mm deep, or 4 $mm^3$. The latter contained *ca.* $5.4 \times 10^{-5}$ millimoles of $NaHCO_3$, and 0.05 mg of fungus produced approximately $3.5 \times 10^{-5}$ millimoles of $CO_2$; a remarkable correlation indeed! The rhizoids of a single R.S. plant cover no more than 0.7 $mm^3$ of agar, and the total $NaHCO_3$ therein would be *ca.* $0.9 \times 10^{-5}$ millimoles!

Two further observations helped to elucidate the mechanism of the $CO_2$ effect: (1) $NaHCO_3$ and $CaCO_3$ did not effect production of R.S. plants on medium B containing 50 $\mu$g thiamine per liter ¹). Furthermore, clusters invariably remained colorless for prolonged periods because the rate of maturation as well as the number of R.S. was greatly reduced. (2) extreme reduction of the atmospheric

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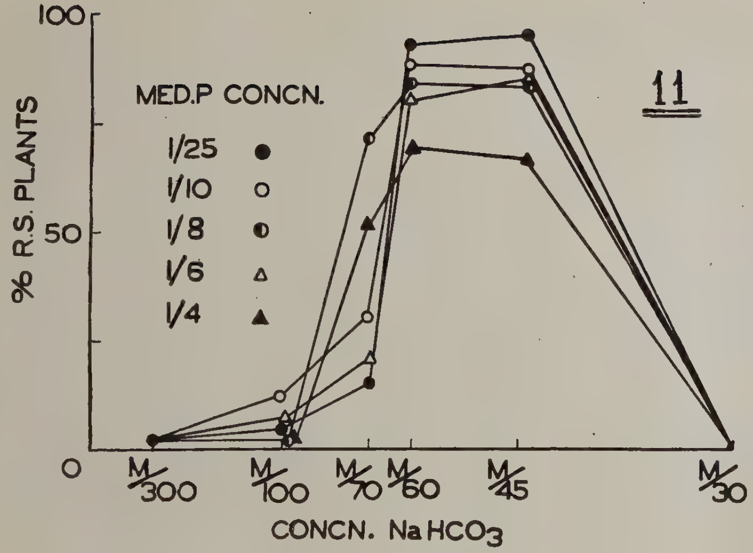
¹) This strain of *Blastocladiella* is heterotrophic for thiamine; see page 340.

**11**



**12**

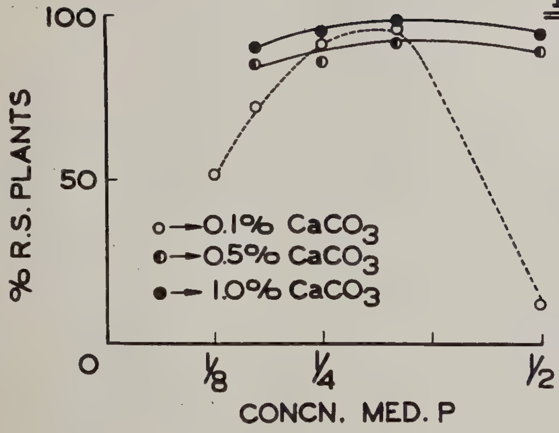


Fig. 11.
The effect of different concentrations of $NaHCO_3$ on the incidence of R.S. plants on medium P at various dilutions. (In this particular instance, medium P was 4 times as concentrated as that indicated under "Materials and methods").

Fig. 12.
The effect of different concentrations of $CaCO_3$ on the incidence of R.S. plants on medium P at various dilutions. (In this particular instance, medium P was 4 times as concentrated as that indicated under "Materials and methods").

$CO_2$ concentration often induced the formation of large quantities (50 to 85%) of R.S. plants on medium P! This was accomplished by placing small containers with 3 cc of 5 N NaOH in the Petri dish cultures, the latter being enclosed in gas chambers containing a 30 cc layer of NaOH at the bottom and continuously flushed out with $CO_2$-free air. To rule out desiccation effects, some plates were placed over $CaCl_2$ and others under reduced pressure; R.S. plants were not produced. What, then, was the morphogenetic mechanism which involved either an increase or a decrease in $CO_2$ concentration, and a factor present in medium P but not in medium B?

Since it had been shown (CANTINO (6)) that the closely-related *Blastocladia* probably fixed $CO_2$ by the Wood-Werkman reaction, *Blastocladiella* spec. might have possessed this capacity. If this were so, and if the tricarboxylic acid cycle operated during genesis of R.S., a mechanism suggested itself. Assuming pyruvate decarboxylation to acetyl phosphate, as well as $CO_2$ fixation *via* pyruvate, and depending upon the individual rates of these two reactions as well as those of oxalosuccinate and *a*-ketoglutarate decarboxylation, either an increase or a decrease in $CO_2$ tension might effect accumulation of the glutarate or some of its precursors back to *cis*-aconitate (Fig. 21). Such compounds, if present in medium P at higher concentrations than in medium B, might directly or indirectly initiate R.S. morphogenesis. Dissociated forms of some Krebs cycle intermediates (e.g. succinate) were not expected to penetrate the thallus of *Blastocladiella* at the pH (*ca.* 7.0) of medium P, but the fact remained that whatever factor(s?) was responsible, individual R.S. plants formed at that pH. Consequently, medium B containing 50 $\mu$g thiamine and 5 g $CaCO_3$ per liter was used as the basal medium for the following studies. Inoculations were made with 0.1 cc of a spore suspension prepared by placing *ca.* 10 to 20 R.S. in 5 cc distilled water and waiting until 70 to 90 % discharge had occurred.

In view of the established or assumed function of vitamins in different steps of, or leading to, the tricarboxylic acid cycle [e.g. pantothenic acid in acetylation (NOVELLI and LIPMANN (26)), thiamine in *a*-ketoglutarate metabolism (BARRON *et al.* (2)) and its established function in decarboxylation, biotin in the Wood-Werkman reaction, and the supposition that it might, by analogy, play a role in the oxidative decarboxylation of *a*-ketoglutaric acid

(Shive and Rogers (30), etc.], 8 of them were tested. Thiamine . HCl, pantothenic acid, riboflavin, pyridoxine, nicotinamide, ascorbic acid, biotin, and *p*-amino benzoic acid, individually and combined, were incorporated in the basal medium at a level of 500 $\mu$g per liter (except biotin, 10 $\mu$g per liter). In addition, to insure that excessively high concentrations in peptone were not responsible, the more suspectable pantothenate, thiamine, and biotin were added individually up to levels of 1000, 1000, and 50 $\mu$g per liter, respectively. Oleic acid and traces of Ni, Cu, and Zn were also tried. In no instance were R.S. plants produced. Next, fumarate, malate, and succinate were tested separately at concentrations up to $8 \times 10^{-3}$ M, but without effect. Succinate, and probably the other two, may have been limited by permeability barriers [cf. Foster (14), p. 362]. Since *Blastocladiella* will not tolerate reactions much below pH 6.0, this difficulty could hardly be circumvented. However, experiments were repeated with agar media bathed in an oxygen-saturated M/45 bicarbonate-carbonic acid buffer at pH 6.3 to 6.5 and contained in stoppered Kolle flasks to prevent loss of $CO_2$. Again, R.S. plants were not produced.

The organic-N content of medium P was appreciably greater than that of medium B; amino acids (glutamic, aspartic, asparagine, and alanine in graded series up to *ca.* $4 \times 10^{-3}$ M) which might by deamination or transamination enter the cycle were therefore tried individually on the basal medium with and without $CaCO_3$ and 500 $\mu$g of pyridoxine per liter. The supposed melanin content of resistant sporangia prompted a similar test with tyrosine. Results were uniformly negative.

Positive results, however, were obtained with *a*-ketoglutarate, which yielded a high incidence of R.S. plants at concentrations of $1.7 \times 10^{-3}$ M and above. In the absence of $CaCO_3$, it did not induce R.S. formation on either the basal medium (brought to pH 7) or medium P. Glutaric acid up to $5 \times 10^{-3}$ M yielded negative results. Oxalacetate, with a half-life of *ca.* one hour, and the more labile oxalosuccinate (Ochoa (27)), were not suitable for such 48 hour growth experiments. Citrate, like *a*-ketoglutarate, also induced formation of R.S. plants (Fig. 13) only in the presence of $CaCO_3$. Up to $5 \times 10^{-3}$ M, *cis*-aconitate, which was expected to behave like *a*-ketoglutarate, did not do so. Pyruvate (Seitz-filtered solutions added after autoclaving), in serial concentrations up to $5 \times 10^{-3}$ M, did not effect their formation when introduced by itself, or with

25 $\mu$g biotin per liter, to the basal medium with or without $CaCO_3$. Acetate, which presumably would yield Krebs cycle intermediates through several channels, gave positive but somewhat inconsistent results (see scattered points, Fig. 13). With or without biotin, it did not induce R.S. formation in the absence of $CaCO_3$. To further insure that this was not due simply to a general effect of organic-acid salts, tartrate, formate, propionate, and butyrate (up to $5 \times 10^{-3}$ M) were tested. Results were uniformly negative for all except butyrate which behaved like acetate. These observations are, in the main, consistent with the original premise (except for the anomalous behavior of *cis*-aconitate). Finally, the high incidence of R.S. plants obtained with added $\alpha$-ketoglutarate was completely eliminated by simultaneously incorporating biotin (25 $\mu$g/liter) in the medium (Fig. 13)! It is noteworthy that this offers indirect but substantial evidence for the possible function of biotin in the oxidative decarboxylation of $\alpha$-ketoglutarate.

Since $CO_2$ appeared to be essential, the problem was further attacked with inhibitors in much the same way that AJL and WERKMAN (1) investigated the substitution of Krebs cycle intermediates for $CO_2$ in the metabolism of *E. coli* and *Aerobacter aerogenes*. Inhibitors were incorporated in the basal medium, with and without $CaCO_3$, as follows: sodium arsenite, 10 concentrations, from $5 \times 10^{-2}$ M to $5 \times 10^{-6}$ M; sodium bisulfite, 5, from $5 \times 10^{-2}$ M to $5 \times 10^{-3}$ M; semicarbazide, 7, from $1 \times 10^{-2}$ M to $2.5 \times 10^{-4}$ M; sodium pyrophosphate, 8, from $5 \times 10^{-2}$ M to $2.5 \times 10^{-4}$ M; Seitz-filtered cyclohexanol, 3, from $5 \times 10^{-1}$ M to $1 \times 10^{-1}$ M, and iodoacetate, 10, from $2.2 \times 10^{-2}$ M to $2 \times 10^{-5}$ M. In the absence of $CaCO_3$, neither cyclohexanol nor bisulfite effected their formation. Pyrophosphate did not cause R.S. production, but induced formation of gametophytes which bore a striking superficial resemblance to young R.S. plants and all of which appeared to be forming R.S. but subsequently discharged *ca.* 24 hours behind schedule. Arsenite and semicarbazide, however, yielded clear-cut results (Fig. 14). Both of these compounds interfere with $\alpha$-ketoglutarate decarboxylation and the results are again consistent with the hypothesis. Iodoacetate reportedly inhibits, among other systems, isocitric dehydrogenase. Added to the basal medium, it induced formation of variable quantities (*ca.* 30 to 60 %) of R.S. plants at concentrations of $4.6 \times 10^{-5}$ M to as low as $2.2 \times 10^{-6}$ M.Although not expressly used as an inhibitor, the behavior of *trans*-aconitate was such that it can best be considered here. At $2.5 \times 10^{-3}$ M, it induced formation of 75 to 95 % R.S. plants; its activity was reduced to *ca.* 30 to 60 % at $1.6 \times 10^{-3}$ M and *ca.* zero at $5 \times 10^{-4}$ M and below. But, in the absence of $CaCO_3$, no growth occurred at concentrations as low as $5 \times 10^{-4}$ M.

The differential effect obtained on media with and without carbonate should be noted (Table 1). In all instances, the concentration required to inhibit growth in the presence of carbonate was higher than that required in its absence.

TABLE 1.

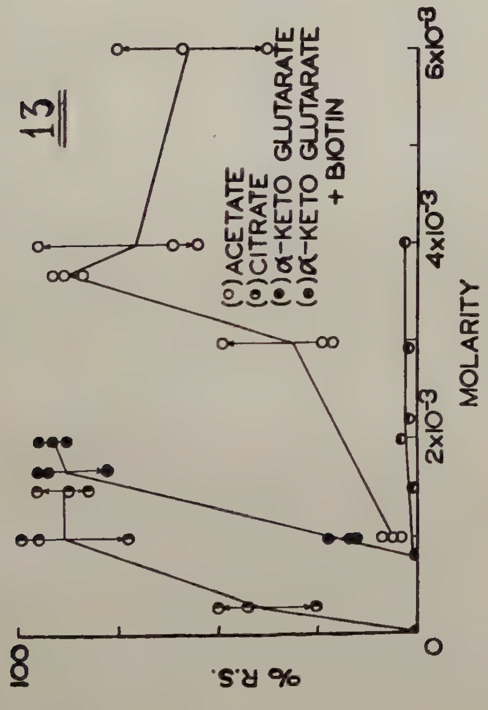
The differential effect of various reagents on growth inhibition of *Blastocladiella* spec. on medium B containing 50 μg of thiamine and with or without 5 g $CaCO_3$ per liter.

| | $CaCO_3$ | | No $CaCO_3$ | |
|---|---|---|---|---|
| | Maximal concentration at which growth occurred | Minimal concentration at which no growth occurred | Maximal concentration at which growth occurred | Minimal concentration at which no growth occurred |
| Arsenite | $2.9 \times 10^{-5}$ M | $5 \times 10^{-5}$ M | None | $5 \times 10^{-6}$ M |
| Bisulfite | $5 \times 10^{-2}$ M | None | None | $5 \times 10^{-3}$ M |
| Semicarbazide | $1 \times 10^{-3}$ M | $2 \times 10^{-3}$ M | $5 \times 10^{-4}$ M1) | $1 \times 10^{-3}$ M |
| Pyrophosphate | $5 \times 10^{-3}$ M | $8 \times 10^{-3}$ M | $5 \times 10^{-3}$ M2) | $8 \times 10^{-3}$ M |
| Cyclohexanol | $2.3 \times 10^{-3}$ M | $5 \times 10^{-3}$ M | None | $1 \times 10^{-3}$ M |
| Iodoacetate | $4.6 \times 10^{-5}$ M | $1.0 \times 10^{-4}$ M | $9 \times 10^{-6}$ M | $4.6 \times 10^{-5}$ M |
| *Trans* aconitate | $2.5 \times 10^{-3}$ M | None | None | $5 \times 10^{-4}$ M |

1) %-spores surviving to produce mature plants was *ca.* 40% at $5 \times 10^{-4}$ M, and *ca.* 100% at $2 \times 10^{-4}$ M.

2) %-spores surviving to produce mature plants was *ca.* 10% at $5 \times 10^{-3}$ M and $2 \times 10^{-3}$ M, *ca.* 50% at $2 \times 10^{-4}$ M, and *ca.* 100% at $1 \times 10^{-4}$ M.

%-survival was based on a comparison with the number of plants on control plates containing no inhibitor, the volume of spore suspension being constant for all experiments; % survival was always *ca.* 100% except as noted.



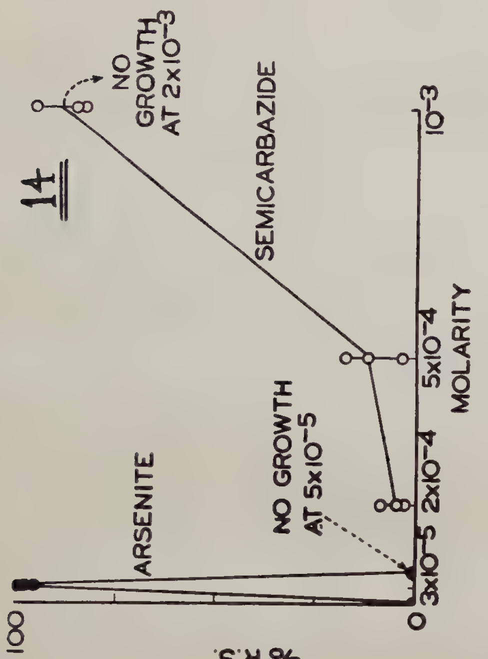


Fig. 13.

The effect of intermediates on the incidence of R.S. plants on medium B containing 50 μg thiamine and 5.0 g $CaCO_3$ per liter.

Fig. 14.

The effect of inhibitors on the incidence of R.S. plants on medium B containing 50 μg thiamine and 5.0 g $CaCO_3$ per liter.

### 4. NUTRITION.

The pH requirements of this strain on solid and liquid media are quite comparable to those of *B. simplex* (EMERSON and CANTINO (12)). Vigorous growth occurs along a roughly-level plateau from pH 6.5 to 8.0, whereas a rapid drop in the curve is evident beginning at 6.0 and growth is generally inhibited at *ca.* 5.5.

It was repeatedly observed that on medium P, plants close together grow faster than isolated ones. For example, on one 24-hour plate, some 75 plants closely appressed to one another averaged 26 × 80 to 160 $\mu$, scattered ones barely 20 $\mu$. On an older plate, values were 32 × 86 *vs.* 150 × 200 $\mu$. A local pH change in the densely populated areas would probably involve a decrease; thus, the differential growth rates can hardly be ascribed to this factor.

Medium P, with or without agar, supported excellent growth. For nutritional studies, however, the more easily standardizable medium B containing 0.3 % glucose was used; with few exceptions (CANTINO (8)), R.S. spore- or gamete-suspensions from single or several R.S. plants and colorless gametophytes grew well on medium B only when thiamine was present at a level of approximately $10^{-1}$ $\mu$g per liter[1]). Subsequently, all inocula were obtained from 3 stock cultures derived from single gametophytes. With these, decreased concentrations induced proportional decreases in absolute growth until, at $10^{-3}$ $\mu$g of thiamine per liter, growth was nil. Nicotinamide, biotin, riboflavin, ascorbic acid, pyridoxine, pantothenate, *p*-amino benzoate, and folic acid did not substitute for thiamine. *Blastocladiella* spec., like *B. variabilis* (QUANTZ (29)), was heterotrophic for thiamine. Growth on medium B plus 3.0 g glucose and $10^{-1}$ $\mu$g of thiamine per liter was then compared with that on medium P with the same amount of glucose. Although the growth rate is lower on the former, final dry weights are roughly comparable. The function of glucose (and starch, which behaves similarly) was not at first clear. Without addition of glucose to medium B, acidic metabolites are not produced, and the growth rate is rather low (Fig. 20). Small quantities of glucose increase the rates of growth and acid production; above 0.3 %, however, the rates again decrease. (Where it is desired to maintain stock cultures without neutralization, media without added carbohydrate are advantageous).

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[1]) Taking into consideration the infrequent but unpredictable and variable content of potentially non-viable swarmers (CANTINO (8)) which was reflected in initial lag periods.

Growth in a completely synthetic medium was not achieved. Some 40 amino acids were combined and substituted for the vitamin-free casein hydrolysate in medium B plus thiamine, but only slight proliferation occurred. Because of possible antagonistic effects among amino acids, glutamate, aspartate, asparagine, cystine, methionine, arginine, and alanine were tested individually and in a few different combinations; the results were also negative. Additional growth factors supplied by the casein may have been necessary, but the question remains unanswered for the present.

The nature of the reactions leading to R.S. formation suggested that if *Blastocladiella* could accomplish the biosynthesis of oxalacetate *via* $CO_2$-fixation, perhaps the thiamine requirement could be partially by-passed by furnishing it with a ready source of $CO_2$. Under these conditions, the supply of acetyl phosphate and its subsequent condensation with oxalacetate were expected to limit synthesis of tricarboxylic acids. With this and recent reports concerning acetylation and Coenzyme A in mind, it was further assumed that the $B_1$ deficiency might be completely by-passed in an environment containing bicarbonate and acetate.

In the presence of pantothenate, this is indeed the case; acetate, bicarbonate, and pantothenate combined can completely substitute for thiamine! Results from triplicate cultures were so sufficiently clear cut that dry weight measurements were not made; representative amounts of growth in the various media are shown in Fig. 10.

Qualitative tests had indicated the presence of lactic acid in cultures growing on glucose media and quantitative data were subsequently obtained. Because of the alternative metabolic mechanisms centering around pyruvate, analytical data were secured with different media (Table 2). It was expected that an excess of biotin and/or acetate might lower the lactic acid yield, but this did not occur. Preliminary data not reported here suggest that coincident with cessation of growth, only 40 to 70 % of the potential lactic acid is formed; the remainder appears thereafter, presumably through glucose dissimilation by the "resting cell suspension", and is possibly indicative of a rate of glucose metabolism lower than and more or less independent of that for "growth". At any rate, approximately 2 moles of lactic acid accumulate for each mole of glucose metabolized. No volatile acids were detected. Neutral volatile and non-volatile compounds were not specifically determined, but their elimination by volatilization and ether extraction did not alter the lactic acid values obtained without such treatments (tested on two cultures only).

TABLE 2.

Lactic acid production by *Blastocladiella* spec. All flasks contained 50 cc medium B plus 3.0 g glucose and 50 $\mu$g thiamine per liter, with additions as indicated.

| | % of carbon, introduced as glucose, recovered as lactic acid | | | | |
|---|---|---|---|---|---|
| | additions to basal medium (per liter) | | | | |
| Replicate cultures | No additions | 5.0 g $CaCO_3$ | 5.0 g $CaCO_3$<br>10.0 $\mu$g biotin | 5.0 g $CaCO_3$<br>10.0 $\mu$g biot.<br>0.16 g acetate | 5.0 g $CaCO_3$<br>0.16 g acetate |
| *a* | 88.0 | 100 | 100 | 105 | 101 |
| *b* | 88.0 | 104 | 106 | 96 | 98 |
| *c* | 86.0 | 104 | | | |
| *d* | 98.0 | 103 | | | |
| *e* | | 111 | | | |
| *f* | | 102 | | | |

## 5. THE GERMINATION OF RESISTANT SPORANGIA.

Germination in this strain of *Blastocladiella* is not significantly different from that of other members of the genus; certain details of cluster R.S., however, require emphasis. Once germination is initiated, several cracks appear in the wall, and these usually coincide with part or the whole length of such ridges; they seem to be involved in a dehiscence mechanism [see SÖRGEL (32) for preformed dehiscence lines in *B. variabilis*]. Several papillae are subsequently formed (very infrequently only one) with hyaline "plugs" averaging 8 × 17 $\mu$. Smaller R.S. (*ca.* 34 × 70 $\mu$), however, particularly when densely packed or partially imbedded in agar media, often form long discharge tubes (*ca.* 13 to 17 × 20 to 24 $\mu$) instead of typical papillae. Larger R.S., too, more or less centrally located in dense clusters, may form long discharge tubes which grow outward irregularly toward open areas. In contrast, resistant sporangia from isolated plants, derived from R.S. spores developing on medium P-$CaCO_3$ plates, possess consistent morphological characteristics; they are always deeply pitted, pigmented, of uniform size, and invariably produce several cracks and several papillae (never tubes and never single papillae) during germination.

It is rather typical of the genus that mature resistant sporangia germinate with relative ease, and the writer's strain is no exception. With the methods developed and outlined in the following pages, R.S. produced on Ca $CO_3$ media at 20° to 22° C. show 100 % germination (without any ill-defined rest periods) approximately one week after spores or gametes are plated out, provided all inhibitory factors described below are eliminated. Though extended tests were not made, 100 % germination was still obtained with 4 month old plates bearing individual R.S. plants. Judging from germination tests with cluster R.S., such sporangia will undoubtedly remain viable for at least one year, and probably for much longer periods.

R.S. produced on carbonate media, particularly suitable for studying germination, were very sensitive to environmental factors. A few examples will suffice to illustrate how variability of germination, so often taken for granted in biological material, may be related to ineffectual (or lack of) control over environmental conditions. Blocks of agar bearing several dozen R.S. plants were placed in water at 20° to 22° C. Percentage germination on different blocks varied from zero to 100 %. Such discrepancy resolved itself in orderly fashion when it became clear that the volume of agar, the volume of water (and whether distilled, tap, or a mixture of the two), and the temperature affected germination. For example: R.S. on agar blocks with a volume of *ca.* 50 $mm^3$ did not germinate in 0.5 cc tap water; 10 to 15 $mm^3$ blocks usually showed 100 % germination. Furthermore, %-germination of R.S. on *ca.* 15 $mm^3$ blocks decreased as the volume of tap water was reduced successively to 0.4, 0.2, 0.1, and 0.05 cc. At certain volume-agar/volume-tap water ratios, R.S. germinated at 20° C. but not at 25° C., even though the latter temperature, *per se*, does not inhibit germination. Each of these observations was related to one general factor; namely, the presence of anions which inhibited germination of otherwise perfectly viable resistant sporangia.

The following studies were made with individual R.S. plants (not cluster R.S.) grown on medium P plus 0.5 % $CaCO_3$. Agar blocks, approximately 6 mm square and 1 mm deep and bearing about

30 to 50 plants, were placed in small Petri dishes containing 5 cc of salt solutions or distilled water.

It soon became clear that inorganic ions had a deleterious effect on germination, and the small quantities normally present in the Delft tap water, together with those in large blocks of agar, could prevent germination (see Table 3 for analysis of tap water).

TABLE 3.

Analysis of Delft tap water (supplied by Mr. D. J. VAN DER VENNEN through the courtesy of the Fabrieken van Gas en Electriciteit en de Waterleiding der Gemeente Delft).

| | |
|---|---|
| Sulfate | $6.9 \times 10^{-4}$ M |
| Chloride | $2.5 \times 10^{-3}$ M |
| Nitrate | $1.1 \times 10^{-4}$ M |
| Bicarbonate | $2.6 \times 10^{-3}$ M |

Furthermore, ions had a differential effect on the cracking of the wall of R.S. as compared to subsequent activity (hereafter simply called germination) leading to discharge of spores. Differential inhibition of cracking and germination is illustrated in Fig. 15. $Mg(NO_3)_2$, $Ca(NO_3)_2$, and $NaNO_3$ inhibited cracking at very low levels (M/350, M/300, and M/250 respectively) but permitted 100 % germination at M/600, M/400, and M/350, respectively. $NaHCO_3$ and $KHCO_3$ were inhibitory at higher levels (M/100 to M/150 and M/100), as were NaCl (M/40) and KCl (M/35 to M/38). Furthermore, although salts inhibited cracking as long as R.S. were bathed by such solutions (up to 4 days in some experiments), there was no permanent effect. Sporangia, washed and transferred to distilled water at any time during this period, invariably yielded 100 % germination. Finally, the data strongly suggest that anions, and not cations, were primarily responsible for the phenomenon.

The effects outlined above, however, are specific for the cracking phase. Resistant sporangia were precracked in distilled water and subsequently transferred to the appropriate solutions. Salts which inhibited cracking at the lower concentrations prevented germination only at much higher levels (e.g. $KHCO_3$, M/35; NaCl, M/12 to M/14; $NaNO_3$, M/10). For the tests with sulfates, which inhibit cracking only at extraordinarily high values, R.S. were not precracked but simply placed directly into the salt solution. $(NH_4)_2SO_4$ and $MgSO_4$ did not inhibit cracking at concentrations as high as M/15 and M/5, respectively, and $Na_2SO_4$ induced inhibition only at M/25 or above. But, germination was prevented at M/30, M/24 to M/25, and M/25 to M/28 respectively. For these three salts, however, microscopic examination of R.S. at concentrations between those causing complete inhibition and those just allowing 100 % germination revealed that the average % discharge from any one resistant sporangium was quite low. At high concentrations, most spores rapidly disintegrated within the sporangium and those liberated behaved similarly (but they did germinate on nutrient media if removed immediately after discharge); at concentrations approaching the 100 % germination value, all were viable.

The same general phenomenon applies for the NaCl and $NaNO_3$ curves, although normal discharge occurred at *ca.* M/22 and above, and M/25 and above, respectively. Observations of plates of medium P, containing different concentrations of $MgSO_4$, $NaNO_3$, and KCl, and inoculated with R.S. spores, offer further substantiation; growth was inhibited at M/25 but not at M/40, M/40 but not at M/60, and M/25 but not at M/50 respectively. Liquid medium P, too, containing $NaHCO_3$ and NaCl at different concentrations and inoculated with spores, supported growth at M/40 but not at M/20 and at M/20 but not at M/10. The differential effect of glucose on the two processes is illustrated in Fig. 19. The aggregate results of these experiments are clearly not explicable in terms of a simple osmotic phenomenon.

It was considered important to know if salts such as $NaNO_3$ interfered with some mechanism essential for all subsequent steps leading to spore discharge, or if, although cracking was inhibited, some of the other processes involved in germination progressed normally. Resistant sporangia were placed in distilled water for different periods and then transferred to concentrations of $NaNO_3$ just causing 100 % inhibition of cracking. When transferred after exposures of up to *ca.* 55 min. in water, at which time cracking normally commences, not one R.S. cracked during the 4-day period that they were observed in $NaNO_3$. Thus, whatever mechanism is involved during the 55 minutes in water, it in no way affects the phenomenon of $NaNO_3$ inhibition. Conversely, R.S. were placed in M/200 $NaNO_3$ for different periods up to 5 hours, washed, and transferred to distilled water. The time required to reach the cracking and spore-discharge stages at 21° C. was the same in all instances.

Further evidence for a distinction between cracking and germination was derived from studies of temperature relations. The data in Fig. 17 show the rate of cracking of resistant sporangia placed in distilled water preheated to different temperatures. Germination values were obtained by pre-cracking R.S. in distilled water at 21° C. for 112 min. and then transferring to water at the desired temperature. Approximate $Q_{10}$ values, using the 50 %-germination points from the graphs for the intervals 15° to 24°, 21° to 30°, and 25° to 35°, are 1.7, 1.6, and 1.7. For cracking, at the intervals 15° to 24° and 21° to 30°, they are 1.4 and 1.1. No cracking occurs at 32° C., the critical temperature being *ca.* 31° C. When cracked at 5° C., and subsequently placed in 20° C. water, R.S. produce papillae; instead of discharging spores, however, the contents usually darken and death presumably ensues. The distinction between cracking and germination is perhaps more clearly seen in Fig. 18 which shows two distinct curves, each with a different slope, maximum, and minimum.

In contrast to the anion effect, where prevention of cracking in no way affects the capacity for subsequent germination, inhibition of cracking by elevated but sub-lethal temperatures affects the rate of cracking and germination (Fig. 16). R.S., placed in water at 35° and 37° C. for different periods and subsequently transferred to water at 21° C., cracked at successively lower rates ¹).

Here too, experiments were done to determine if any processes occurring in the pre-cracking period affect the inhibitory action of 32° and 35° C. temperatures on cracking. Sporangia kept in water at 21° C. for different times up to *ca.* 55 min. and subsequently transferred to water at 32° and 35° C. continued to show 100% inhibition for the 5 days examined. Those transferred after cracking germinate normally, although, at 35° C., many swarmers settle rapidly. At 37° C., results are comparable, but few spores are discharged, and most of these settle and quickly disintegrate.

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¹) Germination values are not recorded because only a small % of R.S. discharge completely after long exposures; the rest cleave out viable spores few of which escape. In general, with exposures of one hour or less, 50% to 100% discharge occurs; exposures of 24 hours or more usually result in little or no discharge.

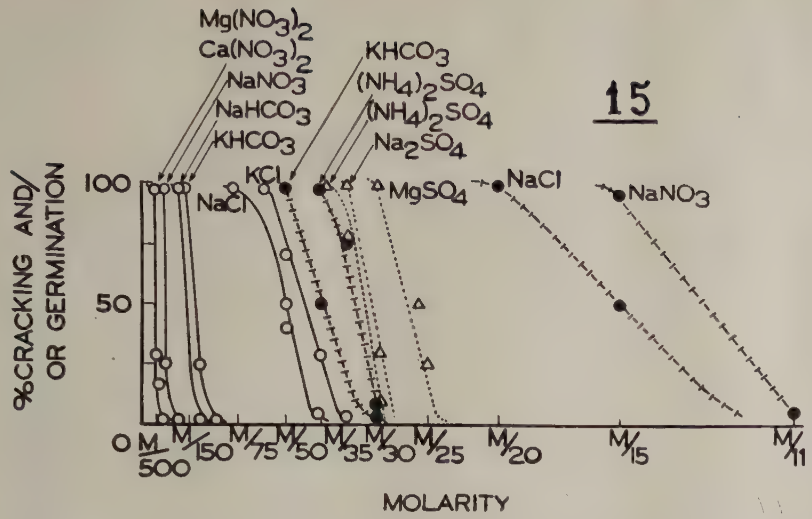


Fig. 15.

The effect of salts at different concentrations on cracking and germination of resistant sporangia. Solid lines: cracking of R.S., cross-hatched lines: germination of pre-cracked R.S., and dotted lines: germination of non pre-cracked R.S.

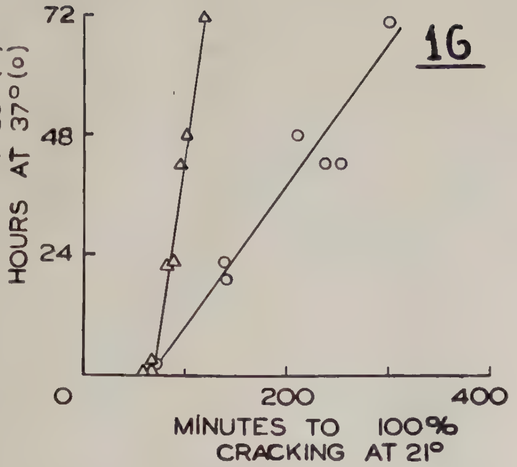
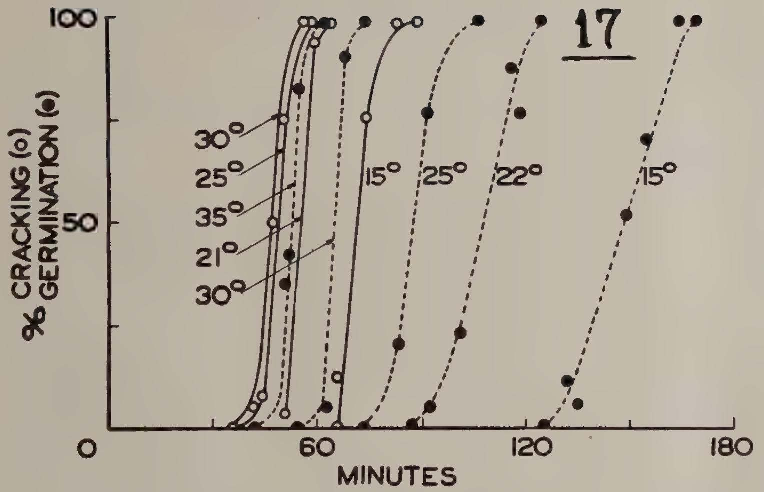


Fig. 16.

The effect of pre-treating R.S. at 35 and 37° C. for different periods of time on their subsequent rate of cracking.



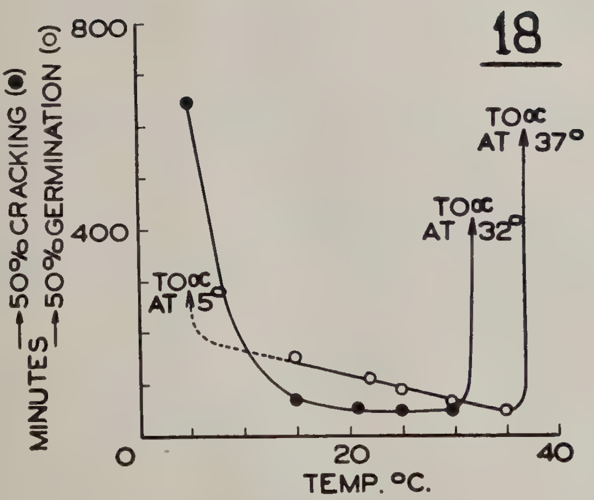
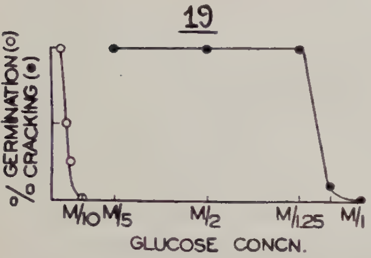


Fig. 17.
The effect of temperature on the rate of cracking of R.S. and germination of R.S. pre-cracked for exactly 112 min. at 21° C.

Fig. 18.
The relation between temperature and cracking and germination, replotted from the data in Fig. 17.



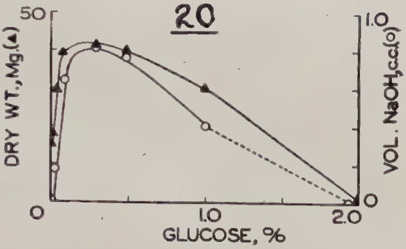


Fig. 19. The effect of different concentrations of glucose on cracking of R.S. and on germination of pre-cracked R.S.

Fig. 20. The effect of different glucose concentrations on growth (dry weight) and detectable (buffered media!) acid production (volume 0.55 N NaOH to maintain the pH at *ca.* 6.0) in 100 cc of medium B after 15 days at 18—19° C.

## DISCUSSION.

### Production of resistant sporangia.

Among the bacteria (KNAYSI (21)), and among the terrestrial Phycomycetes and more particularly perithecial Ascomycetes (HAWKER (20)), sporulation is reportedly affected by one of more of a multitude of factors (salts, pH, carbohydrates, nitrogenous compounds, vitamins, etc.). Relative to the formation of bacterial endospores, KNAYSI concluded (and it is applicable to the situation among the above-mentioned fungi): " . . . various investigators have dealt with each factor as an independent variable. In reality, those factors are so interrelated that the proper evaluation of any one factor is not possible unless its relation to other factors is elucidated". For the aquatic Phycomycetes, however, the seriousness of our confused state of knowledge regarding some aspects of morphology and systematics has recently been brought sharply into focus by EMERSON [(11) pp. 194—198]; it is related to and clearly reflected in the unscientific approaches continuously being made to problems of resting spore production and results derived therefrom. The prevalent notion that resting spores are commonly developed when conditions are unfavorable for zoospore production, for example, may be true, but it is also ambiguous. The comparison is indicative of two rather different approaches to a common fundamental problem.

Finally, even where factors involved in sporulation have been given quantitative meaning, there are few if any instances where their truly biological significance is understood in terms of a fundamental biochemical mechanism. It is believed that such a start has been made with *Blastocladiella*. One phase of morphogenesis in its life cycle has been investigated experimentally and the underlying biochemical transformations associated with it have, in part, been elucidated. Conversely, morphogenesis, *per se*, like oxygen consumption, "growth", etc. has been made to serve as a tool in physiological studies.

The absence of sexual fusions and the variable appearance of clusters made it highly probable that the production of resistant sporangia was primarily under control of environmental factors. It soon became clear that $CO_2$ was one such factor. Submerged in liquid media, the closely related *Blastocladia* [EMERSON and CANTINO (12); CANTINO (6)] forms R.S. only under abnormally high $CO_2$ tensions (*ca.* 100 % $CO_2$); a fact reconcilable with its growth habit among swarms of closely adherent micro-organisms. *Blastocladiella*, which does not form such dense pustules, might be expected to require lower concentrations. This is the case, and in fact it cannot tolerate much more than 5 % $CO_2$.

With *Blastocladiella*, however, gaseous $CO_2$ at concentrations permitting growth never induced more than 50 % (average *ca.* 5 to 25 %) of a population of R.S. spores to form resistant sporangia; furthermore, the results were generally inconsistent. Biologically, $CO_2$ and potential $CO_2$ sources such as $NaHCO_3$ are not necessarily interchangeable, and differential effects might be ascribed, among other things, to permeability phenomena. The very low values for detectable $CO_2$ production by *Blastocladiella* may be significant; perhaps $CO_2$ escapes slowly from the cell because little is produced and/or is rapidly re-utilized, but it may be rapidly re-utilized only because it escapes slowly!

Although the biochemical mechanism underlying the formation of R.S. was approached indirectly, presumptive evidence has been obtained for the existence and possible role of the Krebs cycle during this phase of morphogenesis. The results lend some support, but with reservations, to FOSTER's [(14), p. 411] suggestion that there is no reason to believe, at the moment, that synthesis of tricarboxylic acids in fungi is different from that in other biological entities.

LWOFF and MONOD (24) and AJL and WERKMAN (1) have recently studied the substitution of Krebs cycle intermediates and their precursors for $CO_2$ in the growth of *E. coli* and *Aerobacter aerogenes*; the present investigation dealt with the additive effect of a $CO_2$ source and intermediates in an otherwise fundamentally similar approach.

*Blastocladia* (Cantino (6)) is heterotrophic for thiamine and apparently possesses a poorly developed $C_2$-forming mechanism; during glucose dissimilation, large quantities of lactic acid are produced. Although no detectable $CO_2$ is formed, it appears capable of carrying on a Wood-Werkman condensation.

It is important to re-emphasize the close phylogenetic relationship between *Blastocladia* and *Blastocladiella*. The latter, too, is thiamine-deficient. The thiamine requirement can be partially by-passed with a supply of bicarbonate, and completely so with added acetate and pantothenate, again strongly suggestive of $CO_2$-fixation to yield oxalacetate. The small (but significant) amount of growth in the presence of bicarbonate alone (and plus acetate) may reflect the partial synthesis of enough intermediates for restricted growth which is ultimately limited by the lack of the necessary $C_2$ intermediates. Finally, on glucose media, approximately 2 moles of lactate are liberated for every mole of glucose metabolized. It will be assumed at the start that the pyruvate decarboxylation mechanism, associated with a thiamine biosynthesis that was either lost or never achieved by *Blastocladiella*, is at best a poorly developed one, and a rate limiting step in the initiation of a tricarboxylic acid system (Fig. 21).

Depending upon the factor which becomes limiting for *a*-ketoglutarate decarboxylation in a $CO_2$-free environment, either an increase or a decrease in $CO_2$ tension could induce accumulation of Krebs cycle intermediates between the oxalacetate and *a*-ketoglutarate stages.

The fact that *a*-ketoglutarate induced formation of R.S. plants is consistent with the hypothesis that it, or some precursor, accumulated and was directly or indirectly involved. Furthermore, biotin completely suppressed the phenomenon. This is not only corraborative evidence for the premise, but further strengthens the supposed analogy (Shive and Rogers (30)) between the function of biotin in the Wood-Werkman mechanism and in the oxidative decarboxylation of *a*-ketoglutarate. Though $C_4$ synthesis *via* $C_5$ decarboxylation has not been tested in fungi [Foster (14), pp. 410—411], the mechanism is probably functional in *Blastocladiella*.

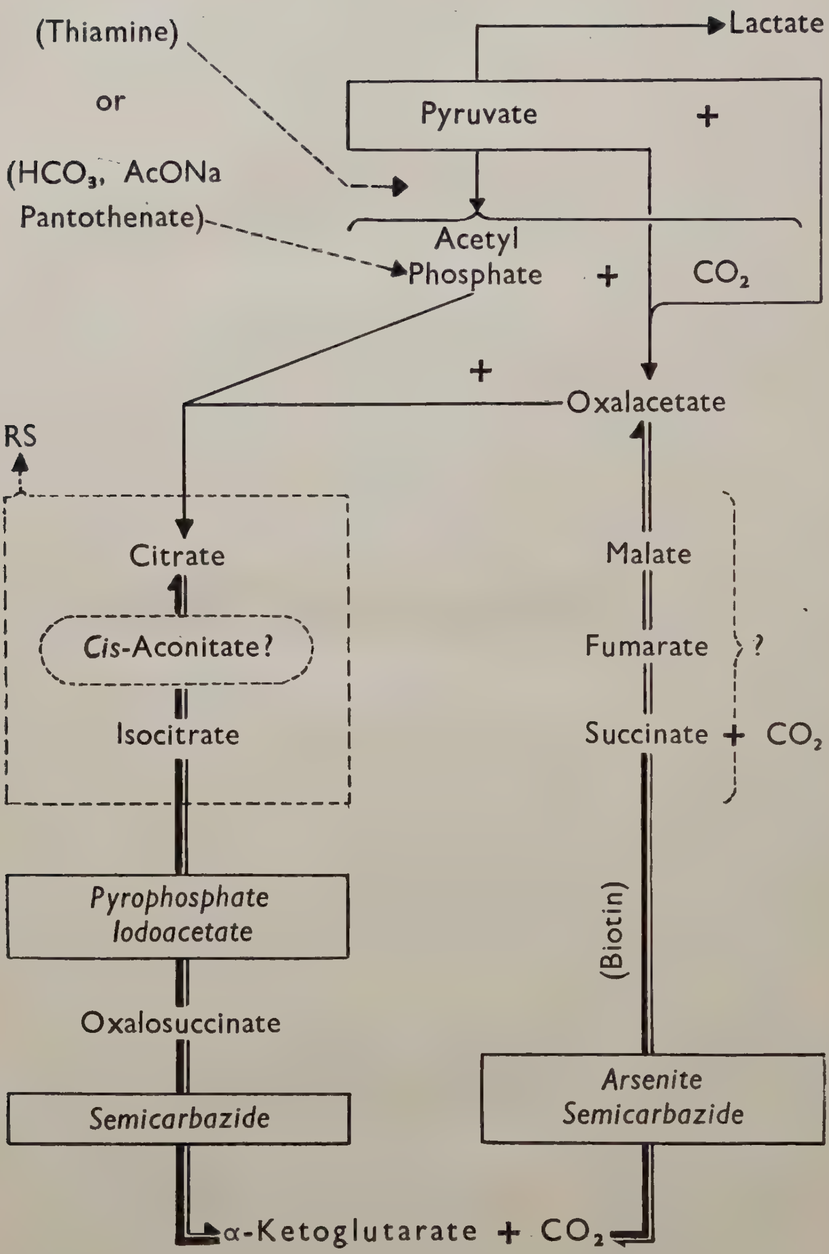


Fig. 21. The possible relation between certain phases of the tricarboxylic acid cycle and morphogenesis in *Blastocladiella*. (cf. context for details).

Arsenite, an inhibitor of oxidative decarboxylation (thus excluding oxalosuccinate decarboxylation), also causes formation of R.S. plants. The hypothesis is again substantiated, with a further implication that *a*-ketoglutarate is not shunted out *via* amination or transamination (or, if it is, at a sufficiently low rate to allow appreciable accumulation). Conversely, glutamate (with or without added pyridoxine), which did not replace the *a*-ketoglutarate, was presumably not deaminated to yield sufficient quantities of the latter compound. Semicarbazide, however, a carbonyl reagent which expectedly combines with such keto compounds, also gave positive results. It may be argued that semicarbazone formation would increase rather than decrease the rate of the reactions leading to production of *a*-ketoglutarate. But, no inconsistency is encountered if it be assumed that formation of a labile enzyme-substrate complex occurs with the semicarbazone as well as the *a*-ketoglutarate. In the absence of permeability barriers, then, added semicarbazone would presumably be as effective as *a*-ketoglutarate in its influence on the morphogenetic pattern; however, experimental proof could not be obtained because of the insolubility of the semicarbazone.

On the other hand, it seems unlikely that semicarbazones of oxalosuccinate or *a*-ketoglutarate could have biological function in initiating R.S. formation. This, together with the instability of oxalosuccinate, suggests that intermediates up to and including isocitrate are more probably implicated. Observations that citrate, too, causes formation of R.S. plants are in keeping with this suggestion. The ineffectiveness of glutarate may have been due to permeability barriers or, more probably, the mold's inability to convert it to the keto derivative.

Pyrophosphate, however, does not induce R.S. formation. Assuming its specificity for isocitrate dehydrogenase, the differential growth inhibition in the presence and absence of $CaCO_3$ is indicative of some degree of accumulation (see below) of isocitrate within the organism. The logical consequence of this supposition is that isocitrate is not the compound specifically responsible for genesis of R.S. Thus, if current concepts of the tricarboxylic acid cycle are applicable here, *cis*-aconitate and iso-citrate are presumably eliminated from consideration. Having ruled out *a*-ketoglutarate and oxalosuccinate, no known intermediate remains. But, the specificity of the inhibitor has been questioned (Krebs (23)). Besides, citrate and to a lesser extent iodoacetate did induce R.S. formation. Finally, it is also possible that accumulation of isocitrate to the critical level could not be attained before toxicity symptoms of the inhibitor appeared; the pronounced morphological changes described support this contention. *Cis*-aconitate, however, was inactive. It is therefore tentatively suggested, contrary to expectations from comparative biochemistry, that *cis*-aconitate is not involved in the tricarboxylic acid cycle during R.S. morphogenesis.

Unfortunately, it is difficult to reach definite conclusions regarding the function of succinate, fumarate, and malate. None induced R.S. formation, but at pH 7, succinate, and probably the others, would not penetrate. Similarly, tests with malonate could not be made because of pH difficulties. If any conclusion can be drawn, it is that this phase of the cycle has no bearing on R.S. formation. Neither aspartate (with or without pyridoxine) which might yield fumarate or oxalacetate, nor cyclohexanol, which reportedly interferes with succinate-fumarate-malate equilibria, gave positive results.

The ineffectiveness of pyruvate, with or without biotin and/or $CaCO_3$, is not inconsistent with the proposed thesis. With a weak $C_2$-forming mechanism, and with appreciable reduction of pyruvate to lactate, it seems likely that any accumulation of oxalacetate, *per se*, would have little effect on the formation of the tricarboxylic acids and their by-products. Alanine, which might deaminate to pyruvate, behaved similarly.

In conclusion, the stimulating effect of $CO_2$ (in medium P) is probably related primarily to the induction of decreased rate of *a*-ketoglutarate decarboxylation and, secondarily, to an increased rate of $CO_2$-fixation *via* oxalacetate. This involves the accumulation of one or more intermediates (citrate, isocitrate?), but at subcritical levels for immediate R.S. formation on medium B. When further supplemented by these intermediates or their precursors, sufficient amounts accumulate to induce R.S. formation. No attempts were made to identify the factors, present in medium P but not in B, which together with $CaCO_3$, influenced the morphogenetic pattern [1]). Since the peptone used was a crude non-standardized preparation, one or more of the postulated intermediates may have been present at sufficiently high levels to account for the results.

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[1]) Compare with papers by LWOFF and MONOD (24) and WYNNE and FOSTER (37) concerning the by-passing of $CO_2$ requirements by substances, other than $C_4$ and $C_5$ dicarboxylic acids, present in complex natural media.

The differential effect of inhibitors in the presence and absence of $CaCO_3$ requires consideration. In every instance, the minimum concentration which induced complete supression of growth in the absence of $CaCO_3$ was lower than that required in its presence. The writer's interpretation corresponds to that of AJL and WERKMAN (1). Briefly, certain concentrations of inhibitor may permit growth in the presence of $CO_2$ sources because the number of molecules of an essential metabolite which may be rendered immobile is determined by the effective concentration of inhibitor within the organism. If, by using $CO_2$ to initiate a flow of such intermediates, they accumulate in excess of the inhibitor, the block will be effectively by-passed and growth will occur. In this light, what can be said concerning the anomalous behavior of *trans*-aconitate, which induced formation of R.S. in the presence of $CaCO_3$ and which affected growth differentially in media with and without carbonate? It should be noted that: (1) the phenomenon can hardly be ascribed to isomerization yielding the *cis* form (unless the *cis*-aconitate did not penetrate and its isomer did); (2) the *trans*-aconitate would presumably be inactive in a tricarboxylic acid system; (3) from special considerations, the *trans*-isomer may be more likely to "fit" the enzyme complex pattern for some Krebs cycle reactions than the *cis*-aconitate. It is, therefore, tempting to speculate that the behavior of *trans*-aconitate is explicable in terms of (competitive) inhibition of some step in the reaction chain from oxalacetate to *a*-ketoglutarate, with consequent accumulation of a metabolite or its precursors involved in R.S. synthesis.

The differential effect, in itself, may allow a rough evaluation of a maximum limit for the quantity of intermediate which must accumulate to effect production of resistant sporangia. For the sake of the calculation, *a*-ketoglutarate is assumed to be the primary metabolite involved. If only the lowest concentration of semicarbazide preventing growth in $CaCO_3$ media (or in $CaCO_3$-less media) were used, its unknown permeability would render calculations impossible. However, it is logical to suppose that the % of inhibitor which actually enters a thallus of *Blastocladiella* would be the same at *ca.* pH 7 in the presence and absence of $CaCO_3$. The difference in minimum concentrations which completely inhibit growth under the two conditions, therefore, is directly related to the total quantity of inhibitor (and therefore *a*-ketoglutarate) which must accumulate per plant before R.S. are formed. For example: from Table 1, the difference between minimal values causing growth inhibition, or those just permitting normal growth, is $1 \times 10^{-3}$ M or $8 \times 10^{-4}$ M, respectively. Assuming the effective volume of agar in contact with the rhizoidal system of the fungus to be *ca.* 1 $mm^3$, a maximum of $10^{-6}$ (or $0.8 \times 10^{-6}$) millimoles of inhibitor and therefore of *a*-ketoglutarate is accumulated per plant to induce R.S. production [1]).

Our knowledge of the metabolism of *Blastocladia* (CANTINO (6)) may, at first glance, appear to introduce a wide gulf between the basis for morphogenesis in it and that in *Blastocladiella*. But, in fact, the data available are not irreconcilable. *Blastocladia* requires a carbohydrate as source of carbon, some 35—54 % of which (as % C) is transformed into lactate and 4—7 % into succinate during growth. With preformed cell suspensions, however, up to 87 % of the carbon is recovered as lactate and 11 % as succinate. But, under the conditions required for production of R.S. (at pH 5.5—5.8 under *ca.* 100 % $CO_2$), the lactate recovery is decreased, the succinate, greatly increased. If decarboxylation of *a*-ketoglutarate is involved, succinate may represent the last stage in a sequence of reactions leading through and resulting in some piling up of components of the tricarboxylic acid cycle. It is significant that malate and fumarate do not support growth of *Blastocladia.*

The two organisms differ fundamentally only in that with *Blastocladia,* fermentable carbohydrates, essential for growth, are carried glycolytically to the pyruvate stage and, only in part, reduced to lactate; the rest of the available carbon is transformed to cell material and succinate but not $CO_2$. *Blastocladiella,* on the other hand, has no absolute requirement for a fermentable carbohydrate; the lack of acid production but formation of detectable $CO_2$ during growth on nitrogenous compounds (e.g. casein hydrolysate) implies that the energetics of the metabolic machine are dependent upon the entry of carbon compounds either (1) into the cycle *via* routes other than pyruvate, or (2) into the cycle through pyruvate under such conditions that no reduction to lactate occurs. Except for the nature and mode of entry of raw materials, the morphogenetic pattern may well have a common biochemical basis.

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[1]) In a recent experiment, a 4 $mm^3$ block of agar medium containing $1.5 \times 10^{-3}$ M semicarbazide and two 4-hour old germlings was transferred to a moist chamber; R.S. plants subsequently developed. Thus, no more than *ca.* $5 \times 10^{-7}$ millimoles of intermediate must accumulate per plant, though the amount may be anywhere below this value.

Lastly, the vitamin deficiency of *Blastocladiella* deserves emphasis. On casein hydrolysate, it is a thiamine heterotroph; on casein hydrolysate-acetate-bicarbonate, it is either a thiamine- or a pantothenate-heterotroph, depending upon which vitamin is tested. In the light of the present work, the results are explicable, but the interpretation of nutritional requirements obviously deserves careful scrutiny [cf. KNIGHT (22), for other examples of this sort among the bacteria]; the bearing of synthetic capacities on phylogenetic relationships among the water molds (CANTINO (7)) may in some cases require further elucidation.

The notion is generally accepted that, in nutritional investigations, the effect of a trace vitamin contaminant *vs.* that of partial autotrophism is reflected in submaximal growth at the normal rate as compared to maximal growth at a subnormal rate, respectively. But, an apparently partial autotroph for $B_1$ might, for example, be in effect completely heterotrophic and grow slowly at a rate limited by diffusion of some other essential metabolite such as $CO_2$ with which the deficiency is by-passed.

### The germination of resistant sporangia.

With the exception of a few fundamental investigations (e.g. GODDARD's (18) contribution on *Neurospora* ascospores), our knowledge of the physiology of spore germination among the fungi is rather nebulous (cf. GOTTLIEB's (19) review). This is particularly true for the aquatic Phycomycetes, where the situation has been frankly evaluated by EMERSON [(11), p. 178]: "Most of the research on resting spores has been of the trial-and-error variety, and too frequently the results reported are difficult to interpret or repeat owing to the use of impure cultures and poorly controlled conditions".

In the genus *Blastocladia*, germination of R.S. produced in pure culture remains a perplexing problem (EMERSON and CANTINO (12)) whereas no such difficulty is generally experienced with *Allomyces* or *Blastocladiella*. Even here, however, consistent and reproducible results have not always been obtained. For example, some of the R.S. of *B. simplex* (MATTHEWS (25)) reportedly germinate if, after a 2 to 3 week rest period, fresh water and food are added to the culture. Similarly, of the R.S. of *B. cystogena* (COUCH and WHIFFEN

(9)), some germinate immediately after their formation and others require a rest period; germination is said to be accelerated by drying for 48 hours or more with subsequent addition of water, or by pouring off the water from the culture medium and then adding more water. In *B. laevisperma*, R.S. germinated only after several weeks of rest (9).

The (abnormal) variability in "rest periods" and germination of R.S. of the Blastocladiales is probably the result of their production under ill-defined conditions. Though not readily admitted by some, to study the factors involved, using gross cultures, is undoubtedly a hopeless task. Even pure cultures, in fact, may still present limitations. For example, resistant sporangia grown by the hundreds over the surface of a hemp seed in pure culture will reach maturity at different times, will be subjected to localized differences in concentration of nutrients and metabolic products with which rhizoids make contact, etc. If such R.S. are selected at random, it should not be surprising to find these conditions reflected in unnecessarily variable results. The generalization is applicable to other sporulating microorganisms, and may offer a partial explanation for the variable 30 hour to 3 month maturation periods required for oospore germination among different species of Saprolegniaceae, the problems encountered in the germination of *Phytophthora* oospores (BLACKWELL (3)), the observations reported by GOTTLIEB [(19), p. 246] that "... with *Sclerotinia fructicola*, as much as 10 hours can intervene between the time the first spore and the last one germinate in a microscopic field", and countless others. There is no *a priori* reason why genotypically homogeneous resting spores, grown under identical conditions, should not have precisely comparable properties. This ideal has been realized with *Blastocladiella* as a result of the discovery that individual well-isolated thalli could be induced to form R.S. plants.

Having achieved suitable conditions for their production, the relation of R.S. germination to environmental factors was established. It is remarkable that salts exert such a pronounced effect and furthermore, that with the exception of sulfate, it is primarily anionic and at such low concentrations that an "osmotic" explanation is hardly tenable. Glucose, at far greater concentrations, offers no inhibition ¹). One may, in fact, wonder if STÜBEN'S (34)

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¹) See, however, FRIES' report [(17) and earlier references therein] con-

inability to obtain germination of R.S. of *B. stübenii* on nutrient agar was due to the salt content of some of his media, and not, as postulated, to an excessively thick wall. When precautions are taken to eliminate all inhibitory environmental factors, the precision with which virtually all R.S. germinate within a few moments of one another is indeed phenomenal. Judging by microscopic examinations of degree of pigmentation, pitting, and wall thickness, R.S. are capable of germination immediately after maturation; there is no suggestion of a rest period. It would be a revelation to know how many watermolds will fit FOSTER and WYNNE's [(15), p. 623] generalization concerning *Clostridium botulinum* that "the so-called "dormancy" in these organisms is not an inherent function of the spores, but rather of the medium in which the spores are placed to germinate".

Differential effects of anions and cations have been thoroughly investigated with other biological materials [cf. PARPART (28), BLINKS (4), WILKINS (36), and many others]. The anion effect in *Blastocladiella* is inexplicable at the moment, but it is now possible to undertake further studies aimed at elucidating the causal mechanism.

One point is perhaps worthy of emphasis. The differential effect of various ions reported here should presuppose caution in studies of the effect of pH on spore germination where, of necessity, buffers consisting of a set of ions in different proportions, or consisting of different kinds of ions at different pH values, are used.

Among mycologists, it is more or less tacitly assumed that germination of resting spores, from the moment they are placed in water until spores are liberated, is a continuous process which involves inward movement of water, subsequent cracking of the wall, and liberation of swarmers. In the bacterial endospore, too, germination (cracking of an inner wall, rupture of an outer elastic one, and liberation of the germ cell) is arbitrarily considered to begin as swelling is initiated and to end as the germ cell emerges (KNAYSI (21)). Among the higher fungi, however, there is some indication (HAWKER (20)) that germination, *per se*, and growth of germ tubes constitute two separate phases; e.g., %-germination may be greatest at one temperature and growth of the germ tubes greater at another, nutrients may have a differential effect on the two processes, etc.

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cerning cationic inhibition (primarily ammonium ions, at *ca.* $10^{-2}$ M) of spore germination in *Mycena*.

The germination of R.S., however, does not include formation of a germ tube, *sensu strictu*, and the elongated papillae which sometimes form and through which spores are discharged have a determinate system of growth; the indeterminate pattern of conidial germ tubes is never encountered. But, it has been shown conclusively that R.S. germination involves a two-step mechanism. Temperature, ions, and carbohydrates exert a differential influence on cracking of the thick pitted wall as compared to subsequent reactions leading to spore discharge. Under normal conditions of germination, cracking is initiated some 55 minutes (at 22° C.) after immersion in distilled water; during this interval, some changes preparatory to spore cleavage and discharge must occur. EMERSON and WILSON (13) have demonstrated that 2N nuclei (in advanced prophase, meiosis I) in the R.S. of *Allomyces* undergo reductional division during the 100 to 130 minute germination period. Yet, in *Blastocladiella*, any internal changes which occur in the 1st. hour can be interrupted, using nitrate, bicarbonate, etc., for hours or days without injury; when transferred to distilled water, germination is resumed on a time schedule as precise as that reported for *Allomyces*.

Finally, many fungal spores germinate with difficulty or are actually inhibited around 35° C. In view of the differential effect of temperature on cracking and germination in this fungus, it might prove profitable to determine if other thick-walled spores, incapable of germinating at certain minimum temperatures, would do so if germination were initiated at room temperatures and then continued at the higher levels.

EMERSON [(11), p. 1] pointed out that there " . . . has come wide recognition of the fact that the aquatic fungi lend themselves very beautifully to the elucidation of certain biological problems of fundamental significance". Believing that the biochemical basis of morphogenesis and problems of spore germination fit this category, *Blastocladiella* spec. would appear to be one such aquatic fungus.

## Summary.

1. One phase of morphogenesis (production of resistant sporangia) in the life cycle of *Blastocladiella* has been investigated experimentally in some detail, and the underlying biochemical transformations have, in part, been elucidated. Conversely, morpho-

genesis has been made to serve as a tool in physiological studies.

Elevated concentrations of $CO_2$ sources induce R.S. spores to develop directly into resistant-sporangial plants instead of gametophytes. The phenomenon, furthermore, is dependent upon additional factors found in a non-standardized peptone, but not in vitamin-free casein hydrolysate. Such factors can be effectively replaced by certain Krebs-cycle intermediates (e.g. $\alpha$-ketoglutarate and citrate) and oxidative-decarboxylation inhibitors or carbonyl reagents (e.g. arsenite and semicarbazide).

Biotin completely eliminates the effect of $\alpha$-ketoglutarate in inducing the formation of R.S. plants; this offers strong indirect evidence for the role of biotin in the oxidative decarboxylation of $\alpha$-ketoglutarate.

*Blastocladiella* is heterotrophic for thiamine. The remarkable fact that it can be replaced by a combination of bicarbonate, acetate, and pantothenate is suggestive of the substitution of a Coenzyme A-dependent acetylation reaction for the decarboxylation of pyruvate during growth.

It has been concluded that retardation of the oxidative decarboxylation of $\alpha$-ketoglutarate, and simultaneous accumulation of its precursors back to the citrate stage, is directly or indirectly related to the formation of R.S. The strong lactate production, the thiamine heterotrophy, the supposedly weak $C_2$-forming mechanism associated with it, the probably-functional Wood-Werkman reaction, and the by-passing of the $B_1$-deficiency with a source of $CO_2$, acetate, and pantothenate are further discussed with relation to the biosynthetic mechanism underlying the morphogenetic pattern.

2. The germination of resistant sporangia, a very precise mechanism, is separable into two distinct phases; one, a period (e.g. 60 min. at *ca.* 20° C.) terminating with cracking of the thick pitted wall, and the other, a subsequent period which leads to the final discharge of spores. Relatively low concentrations of anions, as well as different temperatures, exert a pronounced differential influence on the two processes.

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